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Esterification as a strategy for the detoxification of value compounds in Escherichia coli

Chacon, Micaela

Award date:
2018

Awarding institution:
University of Bath

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**Esterification as a strategy for the
detoxification of value compounds in
*Escherichia coli***

Micaela Chacón

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

September 2017

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Abstract

The use of microbial platforms for the production of commercially valuable compounds has grown over the last few decades as concern over the sustainability of other production methods, such as extraction and chemical synthesis, has been come into question. Advancements in the understanding of microbial metabolism and regulation with the aid of -omics research, and a steady increase in the number of available genetic tools, has allowed model organisms such as the bacteria *Escherichia coli* and the yeast *Saccharomyces cerevisiae* to be engineered for high titre production of a number of value products. The economical production of these compounds on an industrial scale can, however, be hampered by the toxicity of a given end-product upon accumulation in culture. The mode of action by which these products interfere with cellular homeostasis is often multifaceted, making it difficult to address. As such, considerable research has gone into developing strategies to minimize this microbial toxicity in platform hosts; including engineering strains for increased tolerance, and the development of fermentation strategies for the *in situ* removal of toxic end-products. These efforts have had mixed success, with the microbial titres of a number of value compounds continuing to be limited due low tolerance thresholds of the host.

In this work, an alternative strategy for minimizing the toxicity of value alcohols in *E. coli* was investigated. This strategy involved the *in vivo* sequestration of an endogenously produced alcohol into a more neutral short/medium chain ester via enzyme mediated esterification by an alcohol acyl transferase (AAT). The rationale behind this strategy being that the incorporation of a toxic alcohol into a less toxic ester molecule may facilitate higher product accumulation in culture, as the ester end-product would be minimally inhibitory. Further, the alcohol component of the ester could be easily recovered downstream through hydrolysis, while the recovered acid component could be recycled as substrate for this strategy in future cultures. In this work, the feasibility of this detoxification strategy was first validated and then applied to two commercially valuable alcohols in *E. coli*. The first alcohol to which this strategy was applied was the proposed gasoline alternative short chain alcohol, butanol, which is detrimental to culture health when present at concentrations above 1.5% (v/v); and the second alcohol trialled was the common flavour and fragrance constituent monoterpene alcohol, geraniol, which is inhibitory when present above 0.05% (v/v) in culture.

Acknowledgements

I would like to thank my thesis supervisors, Professor David Leak and Dr. Albert Bolhuis, for giving me the opportunity to pursue a stream of research that I truly enjoy. Thank you both for your great insight and guidance throughout this project, and for the flexibility in letting me design my own experiments – some ending successfully, and some ending as horrible failures. I'm a better scientist for having worked with you both. Thank you for taking a chance on me.

I would also like to thank all the member of labs 1.28 and 1.33, past and present, for always being generous with their time and good spirits, and for making my time at the University of Bath and absolute joy. Especially Alex, for being the best friend I could ask for, and for shining some of her charisma, uniqueness, nerve and talent down on me; and Emanuele for being so generous with his optimism, patience (Gibson primers), and time – and for having a drawer full of premium snacks. Also to Jonathan, thank you for somehow simultaneously knocking me off my feet and holding me steady, I love you.

I would like to dedicate this thesis to my family, who have always offered endless support and encouragement. Especially when it involved long nights helping me with homework that I left to the last minute. I have loved every moment watching Stargate SG-1 with you dad, every girly weekend eating almost nothing but candy with you mom, and every time you have accidentally called me Janelle, Benj. Thank you all for everything. In particular, I would especially like to dedicate this to my grandad, who was the kindest person I've ever known.

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Abbreviations

ϵ	Extinction coefficient
μ	Micro
μg	Microgram
μg	Microlitre
$^{\circ}\text{C}$	Celsius
%	Percent
aa	Amino acids
AACS	Acetoacetyl-CoA synthase
AAT	Alcohol Acyl Transferase
ABE	Acetone butanol ethanol
ACP	Acyl carrier protein
ACS	Acetyl-CoA synthase
AdhE2	Aldehyde-alcohol dehydrogenase
ALE	Adaptive laboratory evolution
Amp	Ampicillin
AMP	Adenosine monophosphate
AtoB	Acetyl-CoA acetyltransferase
ATF	Acyltransferase
ATP	Adenosine triphosphate
BAHD	Benzylalcohol acetyl-, anthocyanin- <i>O</i> -hydroxy-cinnamoyl-, anthranilate- <i>N</i> -hydroxy-cinnamoyl/benzoyl-, deacetylvindoline acetyltransferase
bHG	Bovine growth hormone
BMC	Bacterial microcompartment
bp	Base Pairs
CAT	Chloramphenicol acetyltransferase
CoA	Coenzyme A
Crt	Crotonase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fab	Fatty acid biosynthesis
FAEE	Fatty acid ethyl ester
FAD	Fatty acid degradation
FAS	Fatty acid synthesis
FATB1	Fatty acyl thioesterase B1
Fdh	Formate dehydrogenase

FM	Fermentation media
frdBC	Succinate dehydrogenase
g	Gram
GC/MS	Gas Chromatography Mass Spectrometry
GES	Geraniol synthase
GPPS	Geranyl diphosphate synthase
Hbd	3-hydroxybutyryl-CoA dehydrogenase
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
Hyg	Hygromycin
IC	Ion chromatography
ICM	Internal Coordinate Mechanics
IDI	Isopentenyl-diphosphate delta isomerase
IPTG	Isopropyl- β -D-thiogalactopyranoside
ISPR	<i>In situ</i> product removal
Kb	Kilobase
kDA	Kilodalton
L	Litre
LAB	Lactic acid bacteria
LB	Luria broth
ldhA	Lactate dehydrogenase A
LS	Limonene synthase
M	Molar
MCS	Multiple cloning site
MEV	Mevalonate pathway
MEP	Methylerythritol 4-phosphate pathway
Min	Minute
MK	Mevalonate-5-kinase
mL	Millilitre
mol	Moles
mRNA	Messenger ribonucleic acid
MTB	Modified terrific broth
MWCO	Molecular weight cut off
NA	No activity
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NADP	Nicotinamide Adenine Dinucleotide
nM	Nanomolar
nm	Nanometers
NP	Nitrophenyl

NRP	Nonribosomal peptide
OD	Optical density
PCR	Polymerase chain reaction
PFE	<i>Pseudomonas fluorescens</i> esterase
pH	Power of hydrogen
PMD	Mevalonate pyrophosphate decarboxylase
PMK	Phosphomevalonate kinase
POH	Perillyl alcohol
Pta-Ack	Phosphotransacetylase-acetate kinase
RBS	Ribosomal binding site
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rotations per minute
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TAE	Tris-acetate-EDTA buffer
TB	Terrific Broth
Ter	Trans-2-enoyl reductase
TRY	Titre, rate, yield
USD	United States Dollars
WHO	World Health Organization
v/v	Volume/volume
w/v	Weight/volume

1. General Introduction

1.1 Natural products and their biological origin

The natural compounds produced by plants, bacteria, and fungi comprise a broad diversity of structures and functionalities and can serve as a rich pool of molecules for incorporation into food and beverages, flavour and fragrances, pharmaceuticals, industrial chemicals and biofuels (Pickens *et al.*, 2011; Gil-Chavez *et al.*, 2013). These compounds can be divided into two overarching biological groups: primary metabolites and secondary metabolites. Primary metabolites, also called central metabolite, play a key role in maintaining physiological processes and are essential for proper growth and development (Irchhaiya *et al.*, 2015; Gil-Chavez *et al.*, 2013). Examples of primary metabolites include the building blocks for macromolecules such as nucleotides and amino acids, coenzymes such as vitamins, and fermentation products such as organic acids and alcohols (Andrio and Demain, 2010). Secondary metabolites, on the other hand, are not integral to growth and development, but rather play a role in ecological function, including defence and attraction (Wink, 2004; Lee *et al.*, 2013). Examples of these include antibiotics such as penicillin, and odour components such as esters and terpenes. Humans have a long history of utilising both the primary and secondary metabolites produced by plant, fungi and bacteria for their own applications. The consumption of ethanolic beverages such as wine fermented from yeast dates back 7,000 years ago, while the use of plant material for medicine dates back as far as 60, 000 years ago (Fabricant and Farnsworth, 2001; Chamber and Pretorius, 2010).

Plants, specifically, have proven to be prolific producers of commercially valuable secondary metabolites, which can be divided into five groups based on their biosynthetic origin: isoprenoids (terpenes), alkaloids, polyketides, phenylpropanoids and flavonoids (Oksman-Caldentey and Inzé, 2004; Gil-Chavez *et al.*, 2013). The magnitude of chemodiversity produced by plants is a consequence of their immovability, requiring that they develop alternative strategies to mediate functions such as defence against herbivores/pathogens, interaction with pollinators, plant-plant signalling, protection against abiotic stressors etc., (Pickens *et al.*, 2011; Gil-Chavez *et al.*, 2013; Moore *et al.*, 2014). Thousands of these plant products have been

purposed as medications, cosmetics, fragrances, dyes, nutraceuticals and industrial chemicals. Arguable, the most significant contribution plant secondary metabolites have made has been to the pharmaceutical industry, with 11% of the 252 drugs the World Health Organization (WHO) considers basic and essential being exclusively of plant origin, and a significant number of synthetic drugs coming from natural precursors. An excellent example of plant derived medication would be the anti-inflammatory agent, acetylsalicylic acid (commercially known as Aspirin) which is derived from the bark of *Salix alba* L. (willow tree) (Rates, 2001; Der Marderosian and Beutler, 2002; Dias *et al.*, 2012; Liu *et al.*, 2017). Outside of the pharmaceutical industry plant derived compounds have many applications. Compounds such as vanillin and frambinone from orchid pods and raspberry fruit, respectively, are used as flavouring agents in food and beverages, while the fragrant essential oils of a number of flowering species have long been used in cosmetics and perfumes (Sinha *et al.*, 2008; Preedy, 2015). The individual components of these oil mixtures, mostly terpenes, have also been isolated and employed in multiple industries. An example is the monoterpene geraniol, from Palmarosa, which is an ingredient in 76% of deodorants on the European market and 41% of domestic and household products, in addition it is used as an insect repellent, antimicrobial agent and potential anti-cancer drug (Burke *et al.*, 1997; Rastogi *et al.*, 1998; Rastogi *et al.*, 2001; Barnard and Xue, 2004; Chen *et al.*, 2010).

In addition to plants, a number of fungal, and bacterial microorganisms have proven to be a valuable source of secondary metabolites with commercial application – especially as antibiotic agents. Respectively, bacteria and fungi accounting for 70% and 30% of total natural product antibiotics derived from microorganisms. As with plants, these compounds are made by the host to mediate interaction with the surrounding environment, including interspecies competition for resources and predation. Of the bacterially derived antibiotics, an impressive 75% come from actinomycetes, which includes the prolific *Streptomyces* genus (Bérdy, 2005; Pickens *et al.*, 2011). Though many other genera of bacteria – including bacilli, myxobacteria and several marine bacteria – produce a number of complex secondary metabolites with potential for human application (Kim *et al.*, 2016). The largest groups of secondary metabolites produced by bacteria include: polyketides, such as tetracycline and erythromycin, β -lactams such as cephamycin, and nonribosomal peptides (NRPs), such as vancomycin. Fungi, as well, produce a number of valuable polyketides, peptide based compounds, and terpenoids; many of which are medically important, including: the β -lactam antibiotic penicillin, terpenoid cholesterol lowering drug lovastatin, and the cyclic peptide immunosuppressant drug cyclosporine (Hoffmeister *et al.*,

2007; Pickens *et al.*, 2011; Gil-Chavez *et al.*, 2013). In addition to the secondary metabolites produced by bacteria and fungi, a number of their primary metabolites have a long history of commercial use. Microbially produced alcohols (ethanol, butanol, etc.), acids (lactic acid, acetic acid, succinic acid etc.), esters, and phenols are all widely used in the food and beverage industry. These compounds significantly contribute to the intricate tastes and smells of certain products. As well, many of these compounds have further applications as cleaning products, polymer precursor, solvents, fuels, and as bulk and fine chemicals and intermediates (Song *et al.*, 2006; Raspor and Goranovic, 2008; Ghaffar *et al.*, 2014; Moon *et al.*, 2016; Azhar *et al.*, 2017). Commercially available amino acids, such as L-glutamic acid and L-lysine, are also sourced from microbes, with the former being used as a taste enhancer with a unique flavour called 'umami', and the latter as a feed additive (Hermann, 2003).

For a long time, value primary and secondary metabolites were sourced from the host organism in which they are produced via extraction methods. This strategy has often suffered, however, due to supply limitations, low biological concentrations, and the presence of these compounds in conjugates and mixtures (Lam, 2007; Gil-Chavez *et al.*, 2013). In more recent years, with advancements in technology, production of these compounds has branched into synthetic chemistry and engineered microbial hosts in addition to the traditional natural extraction methods.

1.2 Methods for the commercial production of natural products: extraction, chemical synthesis and microbial fermentation

Traditionally, value compounds from plants and microorganisms have been obtained through the extraction of the raw materials, not only of plants, but also of fungi, algae, and bacteria. Strategies for extraction include: solvent extraction, pressurized liquid extraction, subcritical fluid extraction, supercritical extraction, microwave-assisted extraction, ultrasonic-assisted extraction, and distillation (Want and Weller, 2006; Hattab *et al.*, 2007; Plaza *et al.*, 2010; Soria and Villamiel, 2010; Zhang *et al.*, 2011). There are many challenges, however, involved in the extraction of value compounds from natural sources, including (i) low biological concentrations

of the compound(s) of interest, (ii) seasonal variability in the quality of the extract (especially with regards to plant products), (iii) difficulty culturing host species (mostly with regards to fungal and bacterial products), and (iv) potential ecological problems associated with demand, such as raw material shortages (mostly with regards to plant products) (Dubal *et al.*, 2008; Brault *et al.*, 2014; Shaaban *et al.*, 2016; Liu *et al.*, 2017). Together these factors drastically increase the production cost of extraction from natural sources. For example, the ester ethyl butyrate, which has a pineapple-like odour, extracted from natural sources costs approximately 5000\$ (USD)/Kg (Pandy, 1992; Brault *et al.*, 2014). The high cost of extraction methods and increasing product demand lead to the development of alternative strategies for obtaining these value compounds.

With a greater understanding of the chemical structures that constitute these value products, chemical synthesis became a popular strategy for their manufacturing. It has since grown into the primary method by which we obtain these value products, significantly increasing production titres and decreasing both time and cost over raw material extraction. For example, chemically synthesized ethyl butyrate costs a mere 4\$ (USD)/Kg, and can be produced in much larger quantities than what can be obtained through extraction (Pandy, 1992; Dubal *et al.*, 2008). However, there are a number of disadvantages to chemical synthesis, as this method often requires quite toxic solvents and is thus environmentally unfriendly, and lacks substrate selectivity, resulting in undesirable mixtures of regio- and stereo-isomers. Typically, the desired end-product is a single isomer, meaning that this latter issue can result in reduced process efficiency and increased downstream purification costs (Longo and Sanroman, 2006). As well, with regards to flavour and fragrances, United States Code of Federal Regulations (1985) and European Communities (1988) legislations state that ‘natural’ compounds can only be prepared either by extraction from natural sources or by microbial/enzymatic synthesis which involves precursor isolation from nature. Thus, compounds produced through chemical synthesis cannot be labelled as ‘natural’ but rather only as ‘natural-identical’, which has created a dichotomy in the market with consumers preferring products labelled as the former title (Shaaban *et al.*, 2016). The number of drawbacks to chemical synthesis of value products has stimulated interest in making these products via biotechnological means.

In recent years, there has been a large amount of research aimed at developing microbial platforms for the synthesis of value product using fermentation and bioconversion. With better understanding of the metabolic pathways that lead to desired end products, and the tools

necessary for heterologous expression of the relevant genes and pathways in microbial hosts, biotechnology has become a booming business. There are many advantages to bio-production of value compounds over the two strategies described above, including: higher regio- and enantioselectivity of biological enzymes compared to chemical synthesis, lower waste/mild operational conditions than both chemical synthesis and raw material extraction, and accelerated production rates than is possible by raw material extraction of the native host. Moreover, there are a number of compounds that can only be produced via enzymatic means, meaning chemical synthesis is not an option (Johannes *et al.*, 2006; Brault *et al.*, 2014; Shaaban *et al.*, 2016). Microbial production relies heavily on a deep understanding on the enzymatic steps leading to the desired end-product, and requires careful consideration of the choice of heterologous host, as this will significantly influence substrate/cofactor availability, post-translational modifications, organelle targeting, etc. (Fernandez and Vega, 2016). While a relatively new field, it has shown great industrial potential for the sustainable production of a number of value products. However, there are several disadvantages to microbial synthesis of value compounds, including: culture contamination, low titres, and poor titre reproducibility due to a lack of understanding of metabolic control – all of which can be improved upon as tools and comprehension of metabolism advance. Nevertheless, the price of compounds produced via microbial means is still significantly less than that obtained via raw material extraction, for example, bio-produced ethyl butyrate costs 180\$ (USD)/Kg (Pandy, 1992). In addition to being more environmentally sustainably than both raw extraction and chemical synthesis.

1.3 Microbial expression platforms for value product synthesis

The typical workflow for the development of a microbial production platform of a commercially valuable product is (1) choosing a suitable heterologous host, (2) isolation and transfer or requisite genes from native producer into the heterologous host, and (3) optimization of product formation and recovery from the heterologous host (Zhang *et al.*, 2016). With regards to the first step, a number of microbial expression hosts have been developed as platforms for value product formation; these include bacterial, yeast and algal systems. While each of these

platforms are communally advantageous with regards to: high growth rates, ease of culturing and handling, inexpensive growth medium requirements, genetic tractability and traceability (Phulara et al., 2016), they each possess their own individual advantages/disadvantages as heterologous systems. To date, there is no ‘super host’ capable of heterologously expressing all biosynthetic pathways for value product formation, and as such, pathway-microbial host compatibility is crucial. Choice of microbial host should take into consideration whether the desired metabolic pathway can be reconstituted in the heterologous host with regards to: the genetic tools available, the possible need for post-translational modification of heterologous pathway proteins, the availability of biosynthetic precursors and enzymes, compatibility of regulatory systems, the presence of endogenous pathways that may compete or interact with the synthesis of the target product, and the ability of the host to survive under the necessary process conditions to produce the desired end product (Zhang *et al.*, 2008; Chen *et al.*, 2010; Keasling, 2010; Zhang *et al.*, 2016). Often it is the case that metabolic pathways of prokaryotic origin are expressed in prokaryotic hosts, while metabolic pathways of eukaryotic origin are expressed in eukaryotic hosts, as they would be more easily reconstituted in a genetically similar system (Ongley *et al.*, 2013; Alberti *et al.*, 2017). However, there are many exceptions to this.

The most commonly used prokaryotic hosts are: the Gram-negative hosts *Escherichia coli* and *Pseudomonas putida*, and the Gram-positive hosts *Bacillus subtilis* and *Streptomyces* spp. (Wersters *et al.*, 2004; Keasling, 2010; Komatsu *et al.*, 2013; Loeschcke and Thies, 2015). *E. coli* has been described as the workhorse of heterologous expression, and is the most popular bacterial platform organism as it is well characterised and possesses an excellent genetic toolbox (Naurú-Idalia and Bernardo, 2017). For further discussion of the advantages of each individual host strain see Zhang *et al.*, (2016). In addition to these commonly used prokaryotic hosts, several other bacteria have been explored on a smaller scale as potential host platform for value product fermentation because their particular ecological niche is beneficial to industrial production. This may be owing to their ability to grow well in a specific environment (ex. thermophiles, anaerobes), use a variety of alternative feedstocks (ex. pentose sugars, gases), or naturally produce and tolerate high concentrations of end-product. For example, the thermophilic bacterium *Geobacillus thermoglucosidasius* was developed as a platform for ethanol production from cellulose-derived sugars (Cripps *et al.*, 2009). Its ability to grow at high temperatures is a desirable industrial phenotype, as culturing at high temperatures exclude the growth of common mesophilic contaminants, facilitates product recovery for volatile end-products, minimizes cooling costs, and, as intrinsic enzymatic rates are generally higher for

thermostable enzymes, there is higher productivity (Koffas *et al.*, 1999; Frock and Kelly, 2012; Zeldes *et al.*, 2015).

Eukaryotic microbial hosts for heterologous production of value compounds are typically yeasts, though some filamentous fungi have also been employed (Anyagwu and Mortensen, 2015). Much like *E. coli* for bacterial platforms, the yeast *Saccharomyces cerevisiae* is the eukaryotic workhorse for heterologous expression, though other yeasts such as *Pichia pastoris* and *Yarrowia lipolytica* have been used as hosts for value product synthesis – though the former is mostly used as a platform for the production of therapeutic proteins (Zhuang *et al.*, 2015; Sabirova *et al.*, 2011). A significant advantage to using *S. cerevisiae* as a platform host over bacterial systems is its ability to perform post-translational modifications of proteins – including disulfide bond formation, acylation, glycosylation, proteolytic processing, and subunit assembly – which may be required for proper function of pathways originating from eukaryal organisms (Görgens *et al.*, 2004; Liu *et al.*, 2012; Hou *et al.*, 2012; Nielsen, 2013). *S. cerevisiae* is also a tolerant organism with regards to factors such as low pH and high osmotic pressure which makes it suitable for industrial fermentations (Hahn-Hägerdal *et al.*, 2007; Liu *et al.*, 2012). In addition to bacteria and yeast, the unicellular eukaryotic green alga, *Chlamydomonas reinhardtii*, has been proposed as a potential heterologous host (Specht *et al.*, 2010; Meyer *et al.*, 2012). Several benefits to its use include: the ability to grow phototrophically, heterotrophically or using a combination of both; three genomes (nuclear, mitochondrial, chloroplast) each of which can be transformed, and each of which possesses distinct transcriptional, translation and post-translational properties (Meyer *et al.*, 2012). As of yet, algae have been used mostly for commercial protein production, though its industrial applications are branching into value chemical synthesis.

1.4 *E. coli* as an expression platform

E. coli is a Gram-negative facultatively anaerobic bacteria that is commonly found in the intestine of warm blooded animals, and is arguably the most thoroughly studied species of bacteria. Over the years it has been extensively used to investigate the basic mechanisms of

molecular genetics and biochemistry – including our understanding of the genetic code, DNA replication, gene expression, protein synthesis, etc. More recently, as molecular tools have developed and improved, it has also evolved into a preferred platform organism for numerous streams of biotechnology (Singleton, 1999; Cooper and Hausman, 2007). It has a relatively small genome that codes for approximately 4000 different proteins (Blattner, 1997), and this small genome size has been advantageous for genetic analysis. A further advantage to *E. coli* as model organism is the relative ease with which this bacterium can be propagated and studied in the laboratory. It has a rapid growth under well-defined laboratory conditions –being able to divide every 20 minutes given an optimal environment, it is able to reach high cell densities, it is easy to isolate colonies of single cell origin when grown on a semisolid agar containing media, and it has simple nutritional requirements which allows it to live on a wide variety of inexpensive substrates (Cooper and Hausman, 2007). With years of iterative use of this bacterium as a platform for understanding fundamental aspects of molecular biology and biochemistry, it was an obvious choice of host for the first attempt at heterologous expression of a foreign gene – the first example of genetic engineering (Cohen *et al.*, 1973; Chang and Cohen, 1974; Morrow *et al.*, 1974). In the decades since this revolutionary work, there have been a huge number of ‘molecular tools’ developed for *E. coli* to facilitate the heterologous expression not just of single genes, but of whole pathways as well. These tools include: a variety of available expression plasmids – and the ability to create inestimable plasmid permutations with the availability of multiple origins of replication (ori) for different copy number, libraries of promoters, terminators, and ribosomal binding sites that vary in strength, and a number of available selection markers – multiple strategies for the uptake and maintenance of a plasmid by *E. coli*, methods for the genomic integration of a foreign DNA and deletion of endogenous genes, high throughput detection for positive recombinants, online databases and resources that blend genomic, transcriptomic and proteomic data, etc. (Jensen and Hammer, 1998; Martinez-Morales *et al.*, 1999; Lodish *et al.*, 2004; Salis *et al.*, 2009; Padmanabhan *et al.*, 2011). As well, a number of *E. coli* expression strains have been developed for improved heterologous gene expression and protein folding. The modifications made to these strains have included: elimination of specific protease expression, increasing the abundance of uncommon tRNAs, increased tolerance to toxic recombinant proteins, the ability to perform select post-transcriptional modifications, etc (Wagner *et al.*, 2008; Fathi-Roudsari *et al.*, 2016). The choice of *E. coli* expression strain will depend on the origin and nature of the foreign gene(s).

1.5 Metabolic engineering of microbes for improved product formation

After selecting the microbial host to be used as a platform for the synthesis of a given product, the next step involves the expression of the requisite genes from the native producer in the heterologous host. However, it is often not enough to simply express the foreign genes of a metabolic pathway, the development of an economically viable microbial production system often requires extensive optimization of expression to maximize: titre (final concentration in the fermentation media), rate (production per unit of time), and yield (units of product synthesized per unit of raw material consumed), often referred to together as TRY (Stephanopoulos, 2012; Nielsen and Keasling, 2016). This optimization frequently involves not only modulating the expression of the genes composing the foreign metabolic pathway, but also manipulating the genetic and regulatory processes of the host organism in an effort to redirect carbon fluxes towards the desired end-product (Keasling, 2010; Yadav *et al.*, 2012; Nielsen and Keasling, 2016). This practice of maximizing microbial product formation through consideration of whole cell metabolism is referred to as metabolic engineering (Stephanopoulos, 1999; Woolston *et al.*, 2013). Metabolic engineering is a broad multi-disciplinary field that incorporates elements of computational science, chemical engineering, biochemistry, molecular biology, synthetic biology, and genetic engineering to optimize end-product formation in a platform host organism (Yang *et al.*, 1998). In the context of developing a microbial expression platform for the production of a value product via a foreign metabolic pathway, metabolic engineering can be divided into two levels. At the first level is the genetic engineering of the pathway itself to optimize pathway flux, which is to say the rate at which the input metabolite is processed to produce the output metabolite (Koffas *et al.*, 1999; Stephanopoulos, 1999). Often the production of a secondary metabolite is metabolically costly to the cell, and thus native hosts have evolved to produce it in small but sufficient quantities; however, from a biotechnological point of view, these quantities are too low to be economically viable (Parekh, 2009; Pickens *et al.*, 2011). Thus, optimizing pathway flux requires balancing expression of pathway proteins so that they are present at levels that are adequate for efficient transformation of the metabolic intermediates to product at an economical rate, but not so high that their expression impacts host cell health by robbing the cell of substrates and cofactors (Keasling, 2010). As well, the relative expression of proteins within a pathway may require balancing to avoid the formation of bottlenecks and the build-up of intermediates; or the catalytic activity/substrate specificity of one or more pathway proteins may require alteration (Woolston *et al.*, 2013; Fisher *et al.*, 2014). Here, the

use of labelling methods (ex. with the use of ^{13}C isotopic tracers) to measure the flux of intermediates, and the use of enzyme kinetics for protein characterization, are key to elucidating potential pathway bottlenecks. Following this, the available genetic engineering tools for a given host can be used to appropriately modulate pathway flux to streamline product formation (Keasling, 2010; Fisher *et al.*, 2014). However, optimizing of the catalytic steps of a metabolic pathway outside of the wider context of the natural cellular metabolism of the host cell is only a partial success. The second level of metabolic engineering takes into consideration the interface of a given heterologous pathway with host cell metabolism. Often, the interconnectedness of cellular metabolism means that the foreign pathway might be competing with endogenous metabolism for precursors and intermediates. Thus, redirecting carbon flux towards the desired end-product often requires modulation or disruption of native pathways. This can involve genetic modifications aimed at increasing precursor or cofactor supply, minimizing product loss due to unwanted by-product formation, or knocking down competing pathways (Pickens *et al.*, 2011; Wohlleben *et al.*, 2012; Nielsen and Keasling, 2016; Kim *et al.*, 2016). Host cell metabolism can also be harnessed for improved product formation, there are several examples in which expression of a foreign pathway has been intrinsically tied to the maintenance of cellular homeostasis, resulting in a drive towards desired end-product formation (Nissen *et al.*, 2010; Lee *et al.*, 2011).

The discipline of metabolic engineering has benefited greatly from advances in adjacent fields: DNA sequencing has elucidated previously uncharacterised metabolic reactions and identified homologous enzymes and pathways from many different organisms – allowing scientists to create hybrid pathways using genes from multiple sources; advancements in ‘omics’ research, such as genomics, proteomics, metabolomics, and fluxomics help draw the cellular blueprints that are vital to system engineering; new genetic tools allow for more precise and finetuned modifications, while new analytical and computational tools enable researches to both model and *in vivo* track RNA, protein and metabolites in a cell to identify potential bottlenecks and to mine, sort, and analyse biological data to further improve metabolic ‘maps’; and the development of the field of synthetic biology has allowed the synthesis and codon optimization of genes and genetic controls, significantly advancing the rate, and decreasing the cost, at which heterologous pathways can be designed and assembled (Keasling, 2010; Yadav *et al.*, 2013; Woolston *et al.*, 2013; Nielsen and Keasling, 2016). Over the last couple of decades, metabolic engineering has been used to develop cost-effective microbial platforms for the production of fuels, chemicals and pharmaceuticals. The tributaries of this field have extended beyond

streamlining the flux of ‘substrate to product’ of a given metabolic pathway – whether it be in either a native or heterologous host – to all aspects of the fermentation process, from engineering microbial utilization of less expensive feedstocks, to improved end-product recovery strategies (Kumar *et al.*, 1992; Zhang *et al.*, 1995; Pickens *et al.*, 2011; Jeong *et al.*, 2012; Scalcinati *et al.*, 2012; Ledesma-Amaro *et al.*, 2016; Gaida *et al.*, 2016). Through the use of microbial metabolic engineering, a number of commercial products have been brought to market, a few of which are: lysine from *Corynebacterium glutamicum* for use as a feed additive, 1,3-propandiol from *E. coli* for use as a chemical intermediate, and artemisinic acid and isobutanol from *S. cerevisiae* for use as an anti-malaria drug and biofuel, respectively (Tong *et al.*, 1991; Koffas *et al.*, 2003; Martin *et al.*, 2003; Chen *et al.*, 2011; Westfall *et al.*, 2012; Nielsen and Keasling, 2016).

Even with these successes, however, metabolic engineering has not provided us with the ability to produce any desired end-product from a microbial host at economical titres. Factors including global regulatory networks, metabolic burden and intermediate/product toxicity can often impede high level production. The connections between these factors and the genotype is complex and poorly understood. They often involve multiple genes and trickle on effects, making them difficult to target (Woolston *et al.*, 2013). However, as our understanding of a host’s metabolic framework improves, and molecular and synthetic biology tools become more advanced, the number of producible natural and non-natural products will expand and the titres at which they can be produced will increase.

1.6 Production of value compounds in *E. coli*

E. coli is widely employed as a microbial platform host for the production of a number of pharmaceuticals proteins, biofuels, fine chemicals and bulk chemicals in both academic research and industry. *E. coli* was the first heterologous host used for the manufacturing of biopharmaceuticals, with the production of human insulin and bovine growth hormone (bGH) (Baeshen *et al.*, 2014; Baeshen *et al.*, 2015). In the years since, *E. coli* has been used as a platform for the commercial production of a number of therapeutic proteins used in the treatment for hepatitis, osteoporosis, various cancers, etc. (Baeshen *et al.*, 2015). As well, a

significant amount of work has gone into developing *E. coli* as a platform for the production of biofuels as an alternative to the traditional fossil derived transportation fuels. Strain engineering for the production of bioalcohols, such as ethanol, butanol, propanol, and isopropanol has been extensively carried out (Ingram *et al.*, 1987; Atsumi *et al.*, 2008a; Atsumi *et al.*, 2008b; Choi *et al.*, 2012; Koppolu *et al.*, 2016), and more recently, work has been done to develop strains that produce biodiesel (fatty acid methyl/ethyl esters), and hydrocarbons (Nawabi *et al.*, 2011; Choi *et al.*, 2013). The production of fine chemicals, such as various hydroxycinnamic acids, flavonoids, terpenoids, and amino acids for use as pharmaceuticals, food additives and cosmetics has also been a growing research area (Martin *et al.*, 2003; Leonard *et al.*, 2007; Kang *et al.*, 2012; Huang *et al.*, 2013). As has the production of bulk chemicals, such as various diols and organic acids for use as polymer precursors, solvents, detergents, fumigants and antibacterials (Nakamura and Whited, 2003; Thakker *et al.*, 2012; Niu *et al.*, 2014; Jain *et al.*, 2015).

Ultimately, *E. coli* has been developed into a preferred platform organism for the production of a vast range of value compounds. While the process of creating an *E. coli* strain for the synthesis of any given end-product often entails a number of stepwise successes and obstacles, only two specific examples will be further discussed here.

1.6.1 Butanol production in *E. coli*

Reliance on fossil fuels such as petroleum has increased significantly in the twentieth century, and immoderate consumption has led to concern over depletion of reserves in the near future, greenhouse gas emission and pollution, and the cost of petroleum derived products (Alper and Stephanopoulos, 2009; Sun *et al.*, 2015; Koppolu *et al.*, 2016). In order to reduce dependency on petroleum reserves, interest in developing platform for the production of alternative renewable fuels that can be derived through the cellular conversion of biomass to biofuel, has grown. As such, bioalcohols, such as ethanol, propanol and butanol, have become common gasoline additive replacements for use as transportation fuels. Significant success has been achieved for the microbial production of bioethanol, specifically, and it has become the major biofuel

alternative/additive to gasoline. In 2013, approximately 1040 million m³ of ethanol was produced worldwide, more than 80% of which was utilized as biofuel (Peterson and Ingram, 2008; Borah and Mishra, 2011; Sun *et al.*, 2015; Koppolu *et al.*, 2016). One of the key factors that has allowed for the successful development of ethanol producing microbial platforms is the ease with which ethanol is fermented from sugar by a number of microbes, including *E. coli* and *S. cerevisiae*. While bioethanol is often advertised as a major alternative fuel, it does possess some limitations, including low energy density compared to gasoline, high vapour pressure, and high hygroscopicity leading to corrosiveness (Sun *et al.*, 2015). Another bioalcohol, butanol, demonstrates more favourable physical and chemical properties for use as a biofuel when compared to ethanol. Butanol is a C4:0 alcohol and thus possesses an energy content closer to gasoline than ethanol, making it a more economical fuel. It can also be blended with gasoline to higher concentrations than ethanol for use in a standard vehicle engine, has lower water solubility resulting in it being less sensitive to water contamination, and is a less volatile and corrosive fuel than ethanol. These qualities mean that traditional gasoline fuel pipelines would not need to be modified for transporting biobutanol, as would be the case with ethanol fuel (Tracy, 2012; Steen *et al.*, 2008; Lee *et al.*, 2008; Inui *et al.*, 2008; Biobutanol fact sheet, BP). In addition to its use as a biofuel, butanol possesses a number of other commercial applications as an extractant, as a feedstock chemical for many butanol derivatives, and as a solvent (Dürre, 2007; Ezeji *et al.*, 2007; Papoutsakis, 2008; Lee *et al.*, 2008). Thus, the drive to develop economically viable microbial platforms for butanol production is powered by more than just the biofuel industry.

Butanol can be produced microbially via two distinct biosynthetic pathways: the keto-acid pathway and the CoA dependent pathway. In the keto-acid pathway, 2-ketobutyrate – which is derived from the amino acid threonine – is converted to 2-ketovalerate by the action of the LeuABCD enzymes (leucine biosynthesis operon) that compose the norvaline biosynthetic pathway. 2-ketovalerate is then further converted to butanol via a 2-ketoacid decarboxylase (KivD) enzyme and an alcohol dehydrogenase (ADH2) enzyme (Figure 1.1). Via this pathway, approximately 1 g/L of butanol from glucose has been achieved in *E. coli* (Shen and Liao, 2008; Sun *et al.*, 2015).

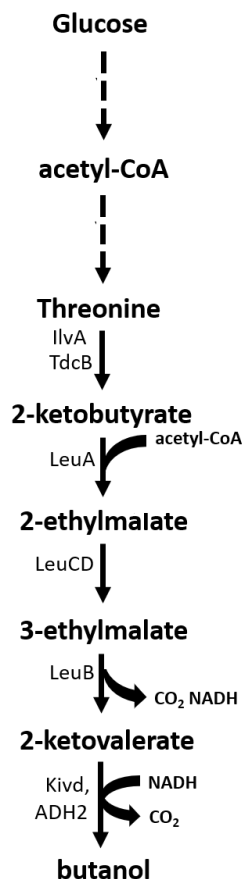


Figure 1. 1 Schematic illustrating the production of 1-butanol via the unnatural norvaline pathway in *E. coli*. The 2-ketobutyrate substrate is derived from threonine.

More successful than the keto-acid pathway for producing butanol is the CoA dependent pathway, which is native to certain solventogenic species of clostridia. This pathway is also referred to as the ABE pathway, as it produces acetone, butanol and ethanol as its end-products in a mass ratio of 3:6:1 (Jones and Woods, 1986; Branduardi *et al.*, 2014). Figure 1.2 shows the ABE pathway, with the genes leading specifically towards butanol shown in black. This butanol producing pathway starts from acetyl-CoA and involves six enzymes: an acetyl-CoA acetyltransferase (Thl), acetoacetyl-CoA thiolase (Hbd), 3-hydroxy-butyryl-CoA dehydrogenase (Crt), butyryl-CoA dehydrogenase (Bcd), electron transfer flavoprotein (Etf), and an aldehyde/alcohol dehydrogenase (AdhE2) (Figure 1.2). Wildtype strains of *Clostridium acetobutylicum* (the most commonly studied butanol producing strain) produce between 12-13

g/L of butanol. However, through the use of genetic and metabolic engineering, achievable butanol titres have reached 18.9 g/L from glucose in fed batch from *C. acetobutylicum*, and 20.5 g/L from mannitol in fed batch from *Clostridia tyrobutyricum* (Lee *et al.*, 2012; Yu *et al.*, 2012). However, compared to *E. coli*, butanol producing species of clostridia are strict anaerobes, have slower

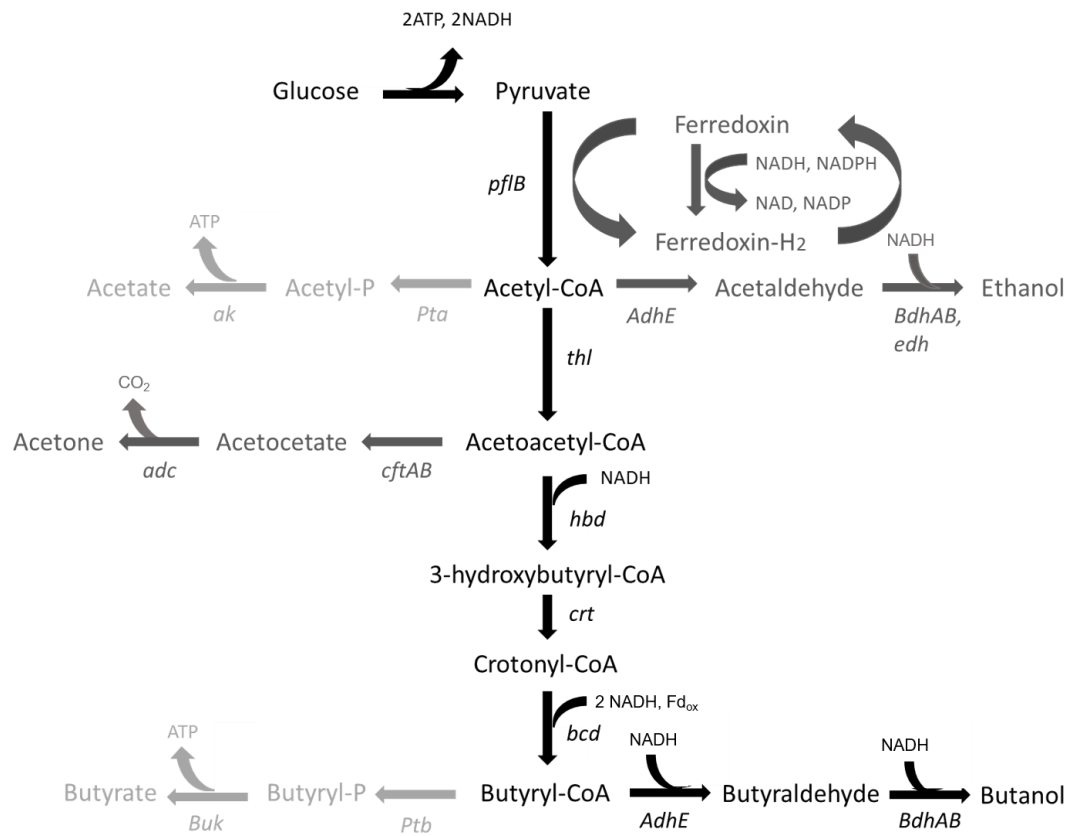


Figure 1. 2 ABE fermentation pathway from *C. acetobutylicum*. Reactions leading towards n-butanol production are in black, while reactions leading to acetone and ethanol production are in dark grey. Enzymes responsible for each reaction are indicated by italic letters next to each arrow.

growth rates, and do not have an abundance of genetic tools available for genetic/metabolic engineering. For these reasons, the CoA-dependent butanol pathway has been reconstituted in more genetically tractable hosts such as *E. coli* and *S. cerevisiae*. The first *E. coli* strain engineered for producing butanol was created by Atsumi *et al.*, (2008), with a final titre of 13.8 mg/L of butanol achieved. Several years of iterative work engineering both the CoA dependent butanol pathway as well as endogenous *E. coli* metabolism to optimize butanol production has so far resulted in maximum titres of 15 g/L from glucose in fed batch cultures – less than the amount that can be produced by engineered clostridia (Shen *et al.*, 2011). In the years following, butanol titres achieved in *E. coli* have not surpassed this value, though interesting work done by Dellomonaco *et al.*, (2011) that same year came close with the production of 14 g/L butanol from glucose in *E. coli* using a strategy that involved the reverse engineering of β -oxidation. A significant impediment to achieving improved titres of butanol in *E. coli* is the high microbial toxicity of this short chain alcohol. Native clostridial producers are only capable of tolerating 2% (v/v) butanol in culture, while *E. coli* strains rarely tolerate more than 1% (v/v) (Fischer *et al.*, 2008; Knoshaug *et al.*, 2009). Butanol toxicity is multifaceted and difficult to address, but has proven to be a central problem to the development of an economical butanol producing *E. coli* platform. As a result, research focussed on identifying the mechanisms of butanol toxicity/tolerance in *E. coli* and other bacteria has intensified in the last few years in an effort to engineer more tolerant strains (toxicity is discussed further in section 1.7) (Ruhl *et al.*, 2009; Mao *et al.*, 2010; Dong *et al.*, 2015). Another tributary of microbial butanol production research in recent years has been aimed at decreasing process costs for *E. coli* fermentation, mostly through optimizing the use of cheap substrates such as glycerol, xylose, palmitic acid, etc. (Dellomonaco *et al.*, 2010; Dong *et al.*, 2015; Saini *et al.*, 2017). Further, work done towards the end of developing a strain of *E. coli* capable of consolidated digestion of plant biomass and butanol production has been trialled using ionic liquid-treated switchgrass as a substrate, and the expression of a cellulase, xylanase, β -glucosidase, and xylobioidase in addition to the CoA-dependent butanol production pathway (Bokinski *et al.*, 2011). While butanol titres achieved from cheaper feedstocks is currently uneconomically low, this work is still in early stages, leaving a lot of room for optimization.

The economics of butanol production from *E. coli* is a fluctuating field, constantly influenced by the current climate. The most significant factor impacting how economical microbial production of butanol (and other biofuels) is the price of oil. Not only as a direct price point competitor to butanol, but also by the more passive means of influencing the amount of interest there is in

biofuel research. There is a correlated increase in interest in biofuel research when oil prices are high, and a decrease when prices are low (Qureshi and Blaschek, 2001). More direct factors influencing the cost of butanol include: type and cost of feedstock, cost of fermentation and product recovery, and the productivity of the microbial strain (Qureshi and Blaschek, 2001; Li *et al.*, 2016; Baral and Shah, 2016). While companies such as Green Biologics Ltd. currently produce butanol via clostridial fermentation, the use of *E. coli* for industrial scale butanol production has not yet been achieved, with further need for improved butanol titres and tolerance.

1.6.2 Monoterpene production in *E. coli*

Terpenes (also called isoprenoids) are the most diverse class of natural product found in plants, fungi, and bacteria, with tens of thousands of reported structures (Lange and Ahkami, 2013; Yamada *et al.*, 2014). All terpenes are built from two or more isoprene (C5) building block. The number of isoprene units from which a terpene is composed is used to classify it, with those that are composed of 10 carbons referred to as monoterpenes (Table 1.2). The volatility of the short chain monoterpenes makes them dominant constituents of plant essential oils, often possessing distinct aromas. They play many ecological roles in both plants and microorganisms, including: attractants for pollinators, broad-spectrum antimicrobials, herbivore deterrents (direct and indirect), and allelopathic agents (Boysen and Hearn, 2010; Tchimine *et al.*, 2013). Monoterpenes have many commercial applications, including extensive use in flavour and fragrances (ex. pinene, geraniol), as anticancer agents (ex. limonene and perillyl alcohol), antiseptics (ex. thymol), insect repellents (ex. citronellol), and their derivatives have application as ‘drop in’ replacements to traditional kerosene aviation jet fuel (van der Werf *et al.*, 1997; Gould, 1997; Lambert *et al.*, 2001; Fortman *et al.*, 2008; Harvey *et al.*, 2010; Brennan *et al.*, 2012; Ryder *et al.*, 2012).

Table 1. 1 Class of terpenes based on backbone chain length.

Terpene	Number of carbons	Example
Monoterpene	10	Geraniol
Sequiterpene	15	Farnesol
Diterpene	20	Taxadiene
Sesterterpene	25	Geranylfarnesol
Triterpene	30	Squalene
Sesquaterpene	35	Ferrugicadiol
Tetraterpene	40	Lycopene

Monoterpenes can be synthesised via two distinct biosynthetic pathways: the mevalonate-dependent pathway (MEV) and the mevalonate-independent pathway (also referred to as the methylerythritol 4-phosphate pathway, or MEP) (Vranová *et al.*, 2013). Higher eukaryotes and some bacteria use the MEV pathway for terpene production, while most prokaryotes and eukaryotic protazoans use the MEP pathway. Plants are an exception to this pathway exclusivity, as they use both pathways in different organelles (Lichtenthaler, 1999, Boucher and Doolittle, 2000; Alonso-Gutierrez *et al.*, 2013). In the MEV pathway, three acetyl-CoAs are condensed successively to produce HMG-CoA, which is then reduced to mevalonate by a reductase. Mevalonate is then phosphorylated twice and decarboxylated to form isopentyl diphosphate (IPP), which can be isomerized to dimethylallyl diphosphate (DMAPP) (Figure 1.3). In the MEP pathway, pyruvate and glyceraldehyde-3-phosphate (G3P) are condensed to form 1-deoxy-D-xylulose 5-phosphate (DOXP), which is further reduced to methylerythritol 4-phosphate (MEP). MEP is then further converted, through several intermediate steps to IPP and DMAPP. The final steps of terpene biosynthesis – which are common to both MEV and MEP pathway– is first the condensation of the IPP and DMAPP precursors by a chain-length specific synthase (Lange and Ahkami, 2013; Figure 1.3). The condensation of one molecule of IPP and one molecule of DMAPP leads to geranyl diphosphate (GPP), the C10 precursor to most monoterpenes. Finally, this GPP can then be converted to a plethora of structurally diverse

monoterpenes through the action of a monoterpene synthase/cyclase (MTPS) (Kinkhead-Reiling *et al.*, 2004; Kuzuyami and Seto, 2012; Lange and Ahkami, 2013; Zebec *et al.*, 2016).

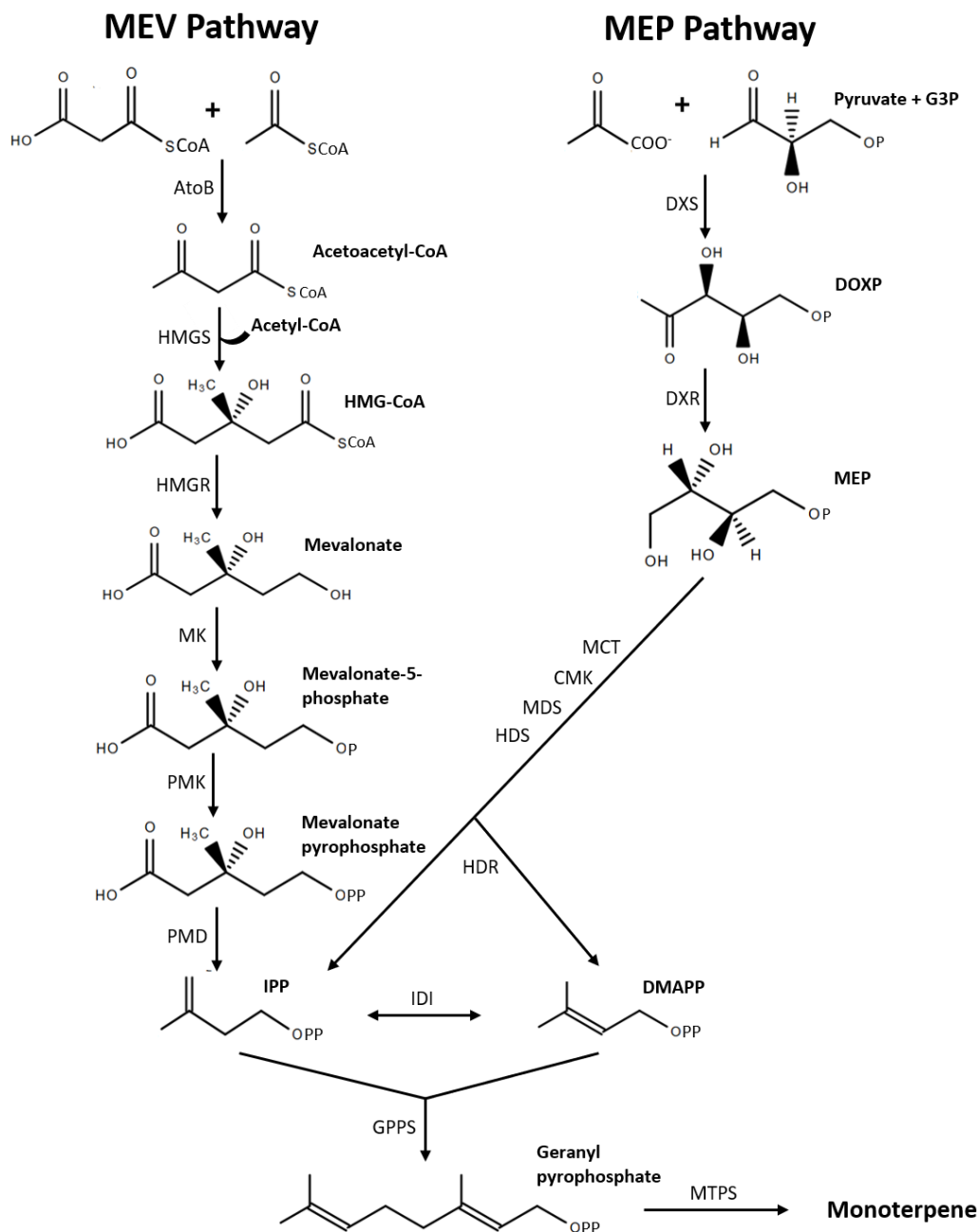


Figure 1. 3 Mevalonate pathway (MEV; left) and non-mevalonate pathway (MEP; right) leading to the production of monoterpenes. Both pathways follow the same reaction sequence after the production of IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate).

As traditional monoterpene extraction from plant material is arduous and expensive, efforts have transitioned to focus on producing these compounds in microbial hosts. A large amount of work has gone into developing *S. cerevisiae* and *E. coli* platforms for the production of a range of monoterpenes. In *E. coli*, strategies involving the engineering of the native MEP pathway or expression of a heterologous MEV pathway have both been employed for monoterpene production (Farmer *et al.*, 2001; Tabata and Hashimoto, 2004; Alper *et al.*, 2005). Of these two approaches, those that involve heterologous expression of the MEV pathway have been more successful, mostly likely because the native MEP pathway in *E. coli* is regulated by unknown mechanism that limit the achievable titres. Endogenous terpene synthesis is intricately tied to cell physiology, and thus far metabolic engineering has not been successful in teasing them apart (Martin *et al.*, 2003). Whether the MEV pathway or native MEP pathway is being employed, monoterpene production in *E. coli* requires the heterologous expression of both a GPPS and MTPS enzyme. Expression of a GPPS is required as *E. coli* does not naturally produce appreciable quantities of geranyl pyrophosphate – the direct precursor to all monoterpenes. While expression of an MTPS is required to dictate the final structure of the monoterpene that will be produced (Kinkhead-Reiling *et al.*, 2004). Monoterpenes such as 3-carene and limonene have been made in *E. coli* through engineering of the native MEP pathway and heterologous GPPS and MTPS expression; though in quite small quantities of 0.003 mg/L and 35.8 mg/L, respectively (Kinkhead-Reiling *et al.*, 2004; Du *et al.*, 2014). In contrast, a larger number of – and higher quantities of – monoterpenes have been produced by *E. coli* through heterologous expression of the MEV pathway. These include α/β pinene, myrecene, sabinene, geraniol, and limonene (Zhou *et al.*, 2014; Zhang *et al.*, 2014; Sarria *et al.*, 2014; Alonso-Gutierrez *et al.*, 2015; Kim *et al.*, 2015). Limonene can be used as an illustrative example of the difference in monoterpene production titres achieved from *E. coli* via either MEP or MEV pathways engineering, with a recent strain engineered with the MEP pathway producing 35.8 mg/L of limonene while a strain engineered with the MEV pathway producing 605 mg/L (Du *et al.*, 2014; Alonso-Gutierrez *et al.*, 2015). Heterologous expression of the MEV pathway in *E. coli* has undergone a number of iterative improvements over the last few years to optimize flux by removing bottlenecks and rate limiting steps in the pathway. Strategies such as gene codon optimization, varying promotor strength, and replacing low activity pathway enzymes with more active homologs from a different organism have been successful in improving end-product titres (Tsuruta *et al.*, 2009; Redding-Johanson *et al.*, 2011; Alonso-Gutierrez *et al.*, 2013).

While developing *E. coli* platforms for the production of monoterpenes is an active field of research, none of the titres achieved to date for any of these compounds has been economic for industrial production – unlike several sesqui- and diterpenes that can be microbially produced in high quantities (Benjamin *et al.*, 2016). A significant impediment towards high titre monoterpene production in *E. coli* is the considerable toxicity these compounds exert in microbial culture (Brennan *et al.*, 2012; Chubukov *et al.*, 2015). Several groups have worked towards elucidating the toxicity mechanisms of these monoterpenes, as well as engineering strains of *E. coli* with increased tolerance to these compounds (Shah *et al.*, 2013; Tomok *et al.*, 2015). As with butanol, however, there has been no major breakthrough to the problem of toxicity to date. As well, a further consideration in the use of *E. coli* as a host for monoterpene production is the source of the MTPS enzyme, as the majority of these proteins have been isolated from plant species. This has implications with regards to the breadth of monoterpenes that *E. coli* may be able to produce given its restricted ability to express eukaryotic proteins. As the number of *E. coli* strains engineered to undertake PTMs increases, so might the library of monoterpenes *E. coli* is capable of synthesizing.

1.7 Obstacles to high titre product formation in microbial platforms – a focus on toxicity

High titre microbial production of value compounds can be hindered at a number of process steps. Product formation often requires engineering of the relevant metabolic pathway and host strain to streamline carbon flux and minimize side product formation in an effort to reach the theoretical maxima of product formation. This process necessitates a balance between heterologous product synthesis and the physiological processes of the host, so as not to impose a cellular burden that negatively impacts titres. Another significant bottleneck in achieving optimal microbial production is toxicity. The number of products being made via microbial platforms consists of: alcohols (ex. ethanol, butanol, fatty alcohols), acids (ex. lactate, succinate, fatty acids), ketones, aldehydes, esters, alkanes/alkenes, and isoprenoids. Additionally, the number of carbon substrates being used for microbial production is also expanding as the hydrolysates of complex carbohydrates sourced from plant biomass or waste are being explored as feedstocks (Nicolaou *et al.*, 2010; Mukopadhyay, 2015). Exposure to unnaturally high levels

of these substrates and products can disrupt cellular homeostasis and result in toxicity in the microbial host. In the case of substrate toxicity, it is usually not the sugars and starches that are inhibitory, but rather the side products and impurities produced during the biomass pre-treatment process that are present in the feedstock (Nicolaou *et al.*, 2010), although high concentrations of pure substrate can cause osmotic stress.

A fundamental necessity for developing strategies for overcoming microbial toxicity is understanding the modes by which a compound acts in the cell to exert toxicity, with compounds that are physiochemically alike often exerting similar effects. With regards to the majority of value compounds produced by engineered microbes, the most significant type of toxicity is organic solvent toxicity. This toxicity is attributed to the accumulation of organic molecules such as alcohols, aldehydes, aromatics, hydrocarbons, etc (Isken and de Bont, 1998; Nicolaou *et al.*, 2010). Many studies have shown that organic solvents impact cell health by (i) imparting physical changes to cell membranes and (ii) damaging biological molecules (VanBogelen *et al.*, 1987; Ramos *et al.*, 1997; Kobayashi *et al.*, 1998; Isken and de Bont, 1998; Ramos *et al.*, 2002; Sardesai and Bhosle, 2002; Volkers *et al.*, 2006). With regards to the former mode of action, solvent-like molecules intercalate into the cell membrane resulting in altered membrane fluidity and function. The cell membrane not only provides structural integrity and a barrier to the extracellular environment, but it is also a matrix in which many enzymes and transport proteins are embedded. Disruption of its integrity results in a number of trickle down effects, including the inability to: maintain turgor pressure, mediate transport in and out of the cell, transduce signals, produce energy, and communicate (Sardesai and Bhosle, 2002; Nicolaou *et al.*, 2010; Mukopadhyay, 2015). With regards to the latter mode of action, impairment to biological molecules can include damage to DNA and lipids via oxidative mechanisms, protein denaturation, and RNA unfolding and degradation (Rutherford *et al.*, 2010; Nicolaou *et al.*, 2010). Here, the extent of damage to a given type of biological molecules depends on the kind of organic solvent. For example, cellular exposure to the short chain alcohol butanol can generate reactive oxygen species (ROS) that damage DNA and lipids, while ethanol exposure results in a chaotropic effect (Woodruff *et al.*, 2013; Chin *et al.*, 2013; Haft *et al.*, 2014).

Cellular response to toxicity is dynamic and can include: (i) induction of metabolic and transport-based detoxification mechanisms, such as metabolism of the toxic compound to one that is less so, or the employment of efflux pumps to remove the toxic compound from the

intracellular milieu; (ii) induction of general and specific stress response systems; (iii) induction of long-term adaptive responses to toxicity, such as alteration of cell membrane composition; or (iv) induction of complex transcriptional and protein level changes that are currently poorly understood (Nicolaou *et al.*, 2010). Each organism, however, has its own intrinsic tolerance level for a given organic solvent, which is determined genetically, as well as being influenced by environmental factors (Huertas *et al.*, 1998; Kobayashi *et al.*, 1998; Sardesai and Bhosle, 2002). For example, *E. coli* is capable of tolerating butanol up to 1.5% (v/v) in culture, while *Pseudomonas putida* is tolerant up to 6% (v/v) (Ruhl *et al.*, 2009; Lamsen and Atsumi, 2012). The high solvent tolerance of non-model organisms is a feature that has been exploited for engineering increased tolerance in platform hosts such as *E. coli*. However, due to the complex nature of tolerance phenotypes, all the genetic determinants dictating tolerance to inhibitory compounds are still not fully understood (Peabody *et al.*, 2014). Ultimately, this lack of understanding has hindered rational engineering of tolerant host strains and no all-encompassing solution to microbial toxicity has been elucidated, although a number of strategies are currently employed with varying degrees of success.

1.8 Strategies for increasing microbial solvent tolerance

Microbial solvent tolerance is not a new field of research; it has been tackled for several applications, including bioremediation for spills and contamination, bio-catalysis in two phase solvent systems, and biotechnology for microbial production platforms of solvent-like compounds (Mukopadhyay, 2015). In the field of biotechnology, research into microbial tolerance is done with the end goal of developing microbial strains where the sensitivity to a desired end-products is alleviated; as this opens the door to higher titre production. Strategies for improving microbial tolerance can be divided to general categories: strain engineering and product sequestration.

1.8.1 Strain engineering

Efforts to rationally engineer microbial strains for increased solvent tolerance are done by upregulating the natural detoxification strategies of the host organism, or by employing strategies/enzymes from species that have been identified as more tolerant to the given stressor. Identification of the genes and pathways involved in microbial detoxification has been significantly assisted by transcriptomic studies of wildtype and solvent exposed microbes (Rutherford *et al.*, 2010; Winkler *et al.*, 2011; Yang *et al.*, 2013; Peabody *et al.*, 2014). From this work, several prominent categories of genes that are involved in tolerance have been identified and exploited to alleviate toxicity in desired microbial hosts. These include chaperones, redox enzymes, transport pumps, membrane-modifying enzymes, and transcription factors/regulators (Dunlop *et al.*, 2011; Ling *et al.*, 2014; Mukopadhyay, 2015). The use of chaperone (proteins which mediate proper protein folding) overexpression to increase solvent tolerance was first carried out in *C. acetobutylicum* with overexpression of the chaperone GroESL. This resulted in a decrease in butanol sensitivity of 85% (Tomas *et al.*, 2003; Papoutsakis, 2008). Overexpression of endogenous chaperones and heterologous expression of thermophilic chaperones in *E. coli* has also improved tolerance to ethanol and a number of other solvent-like compounds (Clark *et al.*, 2008; Zingaro and Papoutsakis, 2012; Abdelaal *et al.*, 2015). Redox enzymes have been overexpressed to address ROS-related protein damage, as it is believed these enzymes act as scavengers for the ‘reactive aldehydes’ produced by lipid oxidation. For example, overexpression of the alcohol dehydrogenase yqhD in *E. coli* improves tolerance to a broad range of compounds, including solvent-like molecules and toxic by-products of biomass pre-treatment (Pérez *et al.*, 2008; Jarboe, 2011; Foo *et al.*, 2014). As well, ROS related stress from butanol exposure was improved in cyanobacteria with overexpression of HspA (Anfelt *et al.*, 2013). Transporters and efflux pumps are a major mechanism for relieving microbial toxicity as they remove the toxic molecule from the intracellular milieu. The RND class of efflux pump (such as AcrAB-TolC), specifically, are heavily involved in conferring solvent tolerance to Gram-negative bacteria (Ramos *et al.*, 1998; Ramos *et al.*, 2002; Segura *et al.*, 2012). *E. coli* mutant strains that displayed increased tolerance to hexane and cyclohexane had upregulated expression of the AcrAB-TolC pump as well as the ABC transporter, ManXYZ (Shimizu *et al.*, 2005; Okochi *et al.*, 2007). While the RND efflux pumps are well characterised for their activity towards antibiotics – and their contribution to antibiotic resistance – they also possess broad substrate range for organic molecules that can range from

hydrocarbons to detergents (Takatsuka *et al.*, 2010). A survey of efflux pumps suggested that the native *E. coli* AcrAB-TolC pump is a potential candidate for improved tolerance towards monoterpenes. Despite its broad substrate range, this efflux pump does not provide tolerance towards more polar compounds, such as short chain alcohol (Ankarloo *et al.*, 2010). Rather, overexpression of ABC transporters instead may increase microbial tolerance to polar compounds; an example is the MdlB transporter in *E. coli*, which when overexpressed improves tolerance to isopentanol (Foo *et al.*, 2014). Strategies that address toxicity upstream of intracellular damage have also been employed successfully. An example of this is maintaining membrane integrity during solvent accumulation by overexpression of lipid modifying enzymes such as desaturases, epoxidases, and cis-trans isomerases (Bernal *et al.*, 2007; Volmer *et al.*, 2014; Jin *et al.*, 2014). Overexpression of these enzymes has been successfully used to maintain membrane rigidity and increase tolerance during solvent exposure. The use of regulators and transcription factors has also been investigated to increase microbial tolerance to solvents. This strategy may prove to be a powerful tool as regulators instigate complex multigenic responses which would be difficult to achieve using individual manipulations (Mukopadhyay, 2015). In cyanobacteria, overexpression of regulators (ex. Srl1037) and transcription factors (ex. SigE) resulted in an increased tolerance to butanol (Kaczmarzyk *et al.*, 2014; Chen *et al.*, 2014); while in *E. coli*, the role of several regulators, including ArcA, has been linked to the butanol and isobutanol stress response – suggesting it may be a future target for tolerance engineering (Brynildsen and Liao, 2009). Ultimately, the use of global regulators may prove to be instrumental for obtaining complex solvent tolerant phenotypes in the future (Mukopadhyay, 2015).

In addition to rational engineering, combinatorial approaches to increasing microbial tolerance have also been performed successfully. This has included (i) the evolutionary engineering of whole strains, as well as (ii) directed evolution of individual proteins involved in the tolerance response. The former involves the direct propagation of the host in inhibitory solvent conditions so that spontaneous mutations that confer increased solvent tolerance can be selected for (Peabody, 2014). This process is referred to as adaptive laboratory evolution (ALE), and benefits from rapid evolution and screening of mutants (Gong *et al.*, 2017). It has successfully been employed to increase the tolerance of microbes to a number of caustic conditions, including an increase in tolerance of *S. cerevisiae* to lactic acid (Fletcher *et al.*, 2017). However, the application of this strategy for improved solvent tolerance has yet to be explored and is likely to be strain specific. The directed evolution of proteins that play a role in microbial

tolerance for higher or more specific activity is a widely used technique. For example, the AcrB protein of the AcrAB-Tol efflux pump complex has been a target of directed evolution to improve its activity towards solvents such as octanol, hexene, and β -pinene (Foo *et al.*, 2013; Mingardon *et al.*, 2015); while global transcriptional metabolic engineering (gTME) – which involves creating randomly mutated libraries of global stress regulators that are then screened for a desired phenotype – has been successfully used in *S. cerevisiae* and *E. coli* to increase tolerance traits (Lin *et al.*, 2013; Si *et al.*, 2014). However, with the redundancy of microbial metabolism, strain engineering for increased solvent tolerance may still not result in economically viable titres. This has led to the development of alternative strategies to minimize toxicity and improve end-product titre that involve *in situ* product sequestration.

1.8.2 Product sequestration

Microbial toxicity can be reduced by limiting exposure of the solvent-like compound to the cells through either *in situ* product removal (ISPR during fermentation or, perhaps, through compartmentalization of the toxic product in organelles. With ISPR, the choice of technique employed will rely on the physicochemical properties of the target compound – taking into consideration its volatility, hydrophobicity, size, and charge (Stark and von Stockar, 2003). A product may be removed from culture by four main techniques. (i) Evaporation via gas stripping, distillation, or pervaporation. This strategy has been successful for short chain alcohols such as ethanol and butanol, with butanol productivity increasing from 0.24 g/L/h to 0.35 g/L/h from *C. acetobutylicum* in a study where gas stripping was used intermittently (Xue *et al.*, 2012). Pervaporation has also been used to overcome butanol toxicity and improve product recovery in cultures of *C. acetobutylicum*, with a productivity increase of 16% using this technique (Xue *et al.*, 2015). (ii) *In situ* extraction into a second phase; this can be done using a water-immiscible organic solvent, or an aqueous two-phase system (Brennan *et al.*, 2012; Iqbal *et al.*, 2016). The use of an aqueous-organic two-phase system has been used extensively for microbial terpene production in *S. cerevisiae* and *E. coli* (Peralta-Yahya *et al.*, 2011; Alonso-Gutierrez *et al.*, 2013; Jongedijk *et al.*, 2015; Liu *et al.*, 2016), as well as being successfully used in free fatty acid producing cultures of cyanobacteria (Kato *et al.*, 2017). (iii) Size selective permeation techniques such as reverse osmosis, dialysis, electrodialysis or nanofiltration.

Dialysis has been used to increase the titre of salicylic acid produced by *Pseudomonas fluorescens* by 20-fold (Abbott and Gerhardt, 1970). And, (iv) immobilization strategies including ion exchange resins, adsorption onto hydrophobic carriers, and affinity absorption. The use of an ion exchange resin has been successfully applied to *in situ* extraction of lactic acid from cultures of *Lactobacillus casei* (Ataei and Vasheghani-Farahani, 2008).

In addition to extracellular sequestration of toxic compounds from microbial cultures to minimize exposure, it may be possible to sequester these molecules internally, either into microcompartments or into less toxic molecules. The former option would involve localizing the biosynthetic pathway of interest to an organelle so that the toxic end-product (or intermediates) would not interact with cellular function (Woolston *et al.*, 2013). This strategy would also have the added benefits of limiting unwanted side reactions by endogenous metabolism, as well as increasing the local concentration of intermediates to potentially drive higher production. While prokaryotes lack true organelles, proteinaceous bacterial microcompartments (BMCs), and other small protein shells do occur naturally (Cheng *et al.*, 2008; Yeates *et al.*, 2010). These BMCs are believed to be involved in encasing sequentially acting metabolic enzymes that catalyse a reaction sequence involving a volatile or toxic intermediate (Cheng *et al.*, 2008). The study of BMCs, however, is relatively new, and a number of questions still remain regarding how the protein shell assembles and selectively allows the movement of certain molecules, but not others, into and out of the compartment (Cheng *et al.*, 2008; Sutter *et al.*, 2017). While preliminary studies have successfully reconstituted functional heterologous BMCs in *E. coli* (Parson *et al.*, 2010; Bonacci *et al.*, 2012), this strategy has not been rationally employed for the sequestration of a metabolic pathway that leads to value end-products that possess high microbial toxicity. However, it represents a potentially powerful tool for microbial detoxification in the future. A simpler strategy for *in vivo* sequestration that has not been explored is the further incorporation of a toxic value compound into a more neutral molecule. Unlike bacterial catabolism of toxic compounds, which typically involves a number of substrate conversion steps – such as toluene breakdown by *P. putida* (Nicolaou *et al.*, 2010), this strategy would incorporate the toxic compound into a more neutral molecule from which it would be easy to retrieve downstream. This is a strategy that bacteria already exploit for the accumulation of poly- β -hydroxybutyrate, but has not been explored for the detoxification of value products in microbial platforms.

Ultimately, the goal of engineering microbial strains for improved tolerance is to increase final production levels. There are a number of examples where engineering strains for improved

tolerance, or end-product sequestration have been successful for increasing final titres. For example, a strain of *S. cerevisiae* engineered for improved salt tolerance also resulted in improved ethanol tolerance and resulted in a dramatic increase in ethanol titres (Lam *et al.*, 2014); while two-phase fermentation has significantly improved the production of styrene in *E. coli* (McKenna *et al.*, 2015). However, while growth and productivity are intimately linked, an improvement in tolerance does not necessarily mean there will be an increase in productivity, as other bottlenecks and pathway regulation may also be limiting improved production (Mukopadhyay, 2015). This stresses the importance of the field of metabolic engineering, which considers a pathway in the entire context of the cell when working towards increasing end-product formation.

1.9 General aims and objectives

The overall aim of this project was to investigate a previously unexplored strategy for reducing the microbial toxicity of heterologously produced value compounds in the platform host, *E. coli*. This strategy involved the *in vivo* sequestration of a toxic end-product into a compound that is more tolerated by the microbial host. Specifically, this work targeted the incorporation of endogenously produced toxic alcohols into less toxic esters via enzymatic esterification in order to evaluate the effectiveness of this strategy for decreasing toxicity/increasing productivity. From this broad aim, a set of objectives were outlined for the project as follows:

- Validation of esterification as a strategy for detoxification of value alcohols in *E. coli* using butanol as a model alcohol.
- Application of this strategy to the short chain alcohol, butanol, in *E. coli*.
- Application of this strategy to the monoterpene alcohol, geraniol, in *E. coli*.

2. Materials and Methods

All chemicals were supplied by Sigma-Aldrich (Gillingham, UK) or Fisher Scientific (Waltham, USA) unless otherwise stated.

2.1 Microbial Growth Media

2.1.1 Luria Broth (LB)

LB media contained 10 g Tryptone (Merk, UK), 5 g Yeast Extract (Melford, UK) and 10 g NaCl dissolved in 1 L of distilled water (dH₂O) and was sterilized by autoclaving (at 121°C and 15 psi for 30 minutes) before use. For LB agar plates, agar was added to a final concentration of 1.5% (w/v) before autoclaving. For antibiotic resistance selection ampicillin was added to a final concentration of 100 µg/mL, kanamycin to a final concentration of 50 µg/mL, tetracycline to a final concentration of 20 µg/mL, and hygromycin (Roche, UK) to a final concentration of 100 µg/mL.

2.1.2. 2X Tryptone Yeast Broth (2TY)

2TY contained 16 g Tryptone, 10 g Yeast Extract, and 5 g NaCl dissolved in 1 L of dH₂O and autoclaved to sterilization before use.

2.1.3 Terrific Broth (TB)

TB media contained 12 g Tryptone, 24 g Yeast Extract, 4 mL Glycerol, 0.17 M KH₂PO₄, and 0.72 M K₂HPO₄ were dissolved in 1 L of dH₂O and autoclaved to sterilization before use.

2.1.4 Modified Terrific Broth (MTB)

MTB media contained 12 g Tryptone, 24 g Yeast Extract, 4 mL Glycerol, and 5 g of NaCl were dissolved in 1 L of dH₂O and autoclaved to sterilization before use.

2.1.5 Fermentation Media (FM)

FM media contained 9.8 g K₂HPO₄, 5 g yeast extract, 0.3 g ferric ammonium citrate, 2.1 g citric acid monohydrate, 0.06 g MgSO₄ and 1 mL of trace element solution which includes 0.37 g (NH₄)₆Mo₇O₂₄·4H₂O, 0.29 g ZnSO₄·7H₂O, 2.47 g H₃BO₄, 0.25 g CuSO₄·5H₂O and 1.58 g MnCl₂·4H₂O) were dissolved in 1 L of dH₂O and autoclaved to sterilization before use.

2.1.6 M9 minimal media

M9 minimal media contained 12.8 g Na₂HPO₄·7H₂O, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 1 mM MgSO₄, 100 µM CaCl₂, 3 nM (NH₄)₆Mo₇O₂₄·4H₂O, 0.4 µM H₃BO₃, 30 nM CoCl₂·6H₂O, 10 nM CuSO₄·5H₂O, 80 nM MnCl₂·4H₂O, 10 nM ZnSO₄·7H₂O, 1 µM FeSO₄·7H₂O, and 0.5% yeast extract were dissolved in 1 L of dH₂O and filter sterilized before use.

2.1.7 Reinforced Clostridia Media (RCM)

RCM media contained 13 g Yeast extract, 10 g peptone, 5 g glucose, 1 g soluble starch, 5 g NaCl, 3 g NaOAc, and 0.5 g cysteine hydrochloride was dissolved in 1 L of dH₂O and autoclaved to sterilize before use.

2.2 *E. coli* strains and plasmids

Table 2. 1 *E. coli* plasmids and strains used in this study.

Name	Description ^a	Source
Plasmid		
pJOE2792	<i>Amp</i> ^R , P _{Rha} , PFE _{Pf}	A gift from Uwe T. Bornscheuer
pET21a::EcAAT	pColE1 ori, <i>Amp</i> ^R , P _{T7} , AAT _{Ec}	This study
pIM8	pCola ori, <i>Kan</i> ^R , P _L lacO ₁ , Ter _{Td}	Atsumi <i>et al.</i> , 2013
pEL11	pCola ori, <i>Amp</i> ^R , P _L lacO ₁ , AtoB _{Ec} , <i>adhE2</i> _{Ec} , <i>crt</i> _{Ca} , <i>hbd</i> _{Ca}	Atsumi <i>et al.</i> , 2013
pBEST-Luc	P15A ori, <i>Amp</i> ^R , P _L lacO ₁ , GFP	A gift from Vincent

		Noireaux (Addgene plasmid # 40019)
pAG32	pBR322 ori, <i>Amp^R</i> , P _{T7} , <i>HygB</i>	A gift from John McCusker (Addgene plasmid # 35122)
pFRANK	p15A ori, <i>Hyg^R</i> , P _{T7} , <i>AAT_{Ec}</i>	This study
pVICTOR	p15A ori, <i>Hyg^R</i> , P _{T7} , <i>AAT_{Ec}</i> , <i>Ter_{Td}</i> , <i>fdh_{Ec}</i>	This study
pVICTOR2.0	p15A ori, <i>Hyg^R</i> , P _{T7} , <i>AAT_{Ec}</i> , P _{T7} , <i>Ter_{Td}</i> , <i>fdh_{Ec}</i>	This study
pADAM	p15A ori, <i>Hyg^R</i> , P _{T7} , <i>AAT_{Ec}</i> , P _{T7} , <i>Ter_{Td}</i> , <i>fdh_{Ec}</i> , <i>fadD_{Ec}</i>	This study
pSHELLEY	p15A ori, <i>Hyg^R</i> , P _{T7} , <i>AAT_{Ec}</i> , P _{T7} , <i>Ter_{Td}</i> , <i>fdh_{Ec}</i> , <i>FatB1_{Cp}</i> , <i>fadD_{Ec}</i>	This study
pET21a::CpFATB1	pColE1 ori, <i>Amp^R</i> , P _{T7} , <i>FatB1_{Cp}</i>	This study
pET28a::RhAAT	pColE1 ori, <i>Kan^R</i> , P _{T7} , <i>AAT_{Rh}</i>	This study
pET28a::GES	pColE1 ori, <i>Kan^R</i> , P _{T7} , <i>GES_{Ob}</i>	This study
pMIB13	p15A ori, <i>Amp^R</i> , P _{lacUV5} , <i>AtoB_{Ec}</i> , <i>HMGS_{Sa}</i> , <i>HMGR_{Sa}</i> , 2T ^b , P _{trc} , <i>MK_{Sc}</i> , <i>PMK_{Sc}</i> , <i>PMD_{Sc}</i> , <i>idi_{Ec}</i> , T1002 ^c , P _{trc} , trGPPS _{Ag} ^d , <i>GES_{Ob}</i>	This study; modified from Alonso-Gutierrez <i>et al.</i> , 2013)
pET21a::Cs2848	pColE1 ori, <i>Amp^R</i> , P _{T7} , 2848 _{Cs}	This study
pET21a::Cs4859	pColE1 ori, <i>Amp^R</i> , P _{T7} , 4859 _{Cs}	This study
pET21a::Cs5680	pColE1 ori, <i>Amp^R</i> , P _{T7} , 5680 _{Cs}	This study
pET21a::Cs2944	pColE1 ori, <i>Amp^R</i> , P _{T7} , 2944 _{Cs}	This study
pET21a::Cs2130	pColE1 ori, <i>Amp^R</i> , P _{T7} , 2130 _{Cs}	This study
pET21a::Cs5781	pColE1 ori, <i>Amp^R</i> , P _{T7} , 5781 _{Cs}	This study
pET21a::Ca2917	pColE1 ori, <i>Amp^R</i> , P _{T7} , 2917 _{Ca}	This study
pET21a::Ca1962	pColE1 ori, <i>Amp^R</i> , P _{T7} , 1962 _{Ca}	This study
pET21a::CaLipG	pColE1 ori, <i>Amp^R</i> , P _{T7} , <i>LipG_{Ca}</i>	This study
Strain	Genotype/plasmids	
BioBlue	K-12 background; <i>recA1</i> , <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44</i> <i>relA1</i> <i>lac</i> [F' <i>proAB</i> <i>lacI^qZΔM15</i> Tn10(Tet ^r)]	Bioline
DH5α	<i>fhuA2</i> <i>lac</i> (del)U169 <i>phoA</i> <i>glnV44</i> Φ80' <i>lacZ</i> (del)M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	Thermo Fisher
C43 (DE3)	F – <i>ompT</i> <i>hsdSB</i> (<i>rB</i> - <i>mB</i> -) <i>gal</i> <i>dcm</i> (DE3)	Lucigen
JCL166	BW25113/F' [<i>traD36</i> <i>proAB</i> ⁺ <i>lacI^qZΔM15</i> (Tet ^r)] <i>ΔldhA</i> <i>ΔadhE</i> <i>ΔfrdBC</i>	Atsumi <i>et al.</i> , 2008
JW5020	F-, <i>Δ</i> (<i>araD-araB</i>)567, <i>ΔfadE739::kan</i> , <i>ΔlacZ4787(::rrnB-3)</i> , <i>λ</i> , <i>rph-1</i> , <i>Δ</i> (<i>rhaD-rhaB</i>)568, <i>hsdR514</i>	Baba <i>et al.</i> , 2006 Coli Genetic Stock Center (New Haven, Connecticut)

DLB	JCL166 pEL11 + pIM8	This study
DLBO1	JCL166 pEL11 + pVICTOR	This study
DLBO2	JCL166 pEL11 + pIM8 + pFRANK	This study
DLG2	C43(DE3) pMIB13	This study
DLGA3	C43(DE3) pMIB13 + pET28a::RhAAT	This study

^a in plasmid description, subscripts indicate the source of the gene as follows: Pf, *Pseudomonas fluorescens*; Ec, *Escherichia coli*; Td, *Treponema denticola*; Ca, *Clostridia acetobutylicum*; Cp, *Cuphea palustris*; Rh, *Rosa hybrid*; Sa, *Staphylococcus aureus*; Sc, *Saccharomyces cerevisiae*; Ab, *Abies grandis*; Ob, *Ocimum basilicum*; Cb, *Clostridia saccharoperbutylacetonicum*

^b in plasmid description, antibiotic abbreviations are as follows: Amp, Ampicillin; Kan, Kanamycin; Hyg, Hygromycin; Tet, Tetracycline

^c 2T: both the rrnB T1 terminator and T7Te terminator

^d T002: terminator

^e tr-GPPS: truncated sequence of GPPS

2.3 Molecular biology methods

2.3.1 Codon harmonization and optimization

The alcohol acyltransferase 16 (AAT16) gene from *Actinidia chinensis* was codon harmonized to *E. coli* using codon usage tables. The *A. chinensis* codon usage table was created using DAMBE (Data Analysis and Molecular Biology and Evolution, Xia, 2013). The harmonized sequence was subsequently termed EcAAT16. All other synthesized genes were codon optimized to the genome of *E. coli* using GeneArt software (Thermo Fisher Scientific, UK).

2.3.2 Acquisition of bacterial knockout strains, genomic DNA, and harmonized/optimized gene sequences

The genomic DNA from *clostridium acetobutylicum* and *C. saccharoperbutylacetonicum* was purchased from the DSMZ collection catalogue (Germany). The *E. coli* knockout strain JW5020 (Δ FadE) was obtained from the Coli Genetic Stock Center (CGSC) (Connecticut, USA). All optimized and harmonized genes were synthesized by GeneArt.

2.3.3 Polymerase chain reaction (PCR)

2.3.3.1 End point PCR

PCR reaction were carried out in 0.25 mL PCR tubes using a final liquid volume of 50 μ l composed of: 1 μ l of template DNA, 5 μ l of each of the 10 μ M primers, 5 μ l of 2.5 mM dNTPs, 10 μ l of 5X Phusion HF-buffer, 19 μ l of Milli-Q water (18.2 m Ω), and 1 unit Phusion Hot Start II polymerase. PCR reactions were performed according to the manufacturer's instructions for 30 cycles. The annealing temperature was varied depending on the T_m of the specific primers used for the given reaction. Reactions were performed using a Bioer Genepro thermal cycler (Alpha Laboratories, UK).

2.3.3.2 Point mutation PCR for site-directed mutagenesis

PCR reactions were carried out as described in section 2.3.1.1 for end point PCR, in two sequential DNA amplifications. Figure 2.1 demonstrates the two-step process. The first amplification consisted of flanking primers (A & B) that were complementary to the ends of the target sequence, and internal primers (C & D) that contained a mismatch capable of generating a mutation corresponding to the desired amino acid change. The first PCR reactions involved creating fragments AD and BC, each of which contain the desired point mutation. The second PCR reaction involved using both fragments AD and BC as a template and using the flanking primers (A & B). The complementary ends of both templates hybridize allowing initial fragment extension followed by PCR amplification of the full sequence, creating a final single product containing the desired internal point mutation. A modification to the second PCR reaction involved using a larger volume of equimolar amounts of fragments AB and CD than described above in section 2.3.1.1. The PCR reaction contained: 10 μ l of fragment AB, 10 μ l of fragment CD, 5 μ l of each of the 10 μ M primers, 5 μ l of 2.5 mM dNTPs, 10 μ l of 5X Phusion HF-buffer, and and 1 unit Phusion Hot Start II polymerase.

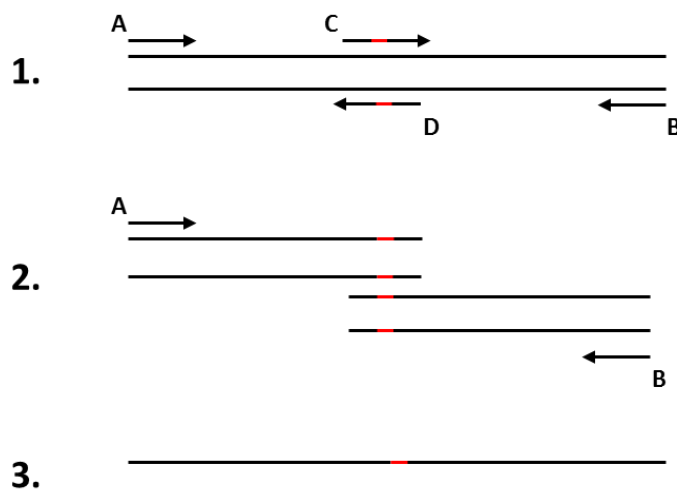


Figure 2. 1 Site-directed mutagenesis by primer extension.

2.3.3.3 Diagnostic/colony PCR

PCR reactions were carried out in a final volume of 25 μ l composed of: 1.5 μ l of each of the 10 μ M primers, 12.5 μ l of 2X KAPATaq ReadyMix, and 9.5 μ l of Milli-Q water (18.2 m Ω), and a *E. coli* colony added directly to the mix. PCR reactions were performed according to the manufacturer's instructions for 35 cycles, with the annealing temperature varying depending on the T_m of the specific primers used (Appendix).

2.3.4 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

2.3.4.1 Isolation of RNA from *E. coli* cells

Overnight cultures of *E. coli* strain C43 (DE3) harbouring either no plasmid, pFRANK or pVICTOR2.0 were inoculated into TB + 2% glucose to 1% (v/v) and incubated at 37°C and 250 rpm until the OD₆₀₀ reached ~1. Each culture was then induced with 0.4 mM IPTG before continuing incubation at 37°C for a further 16 hours. RNA was isolated from 1 mL culture samples using the GenElute™ Total RNA purification Kit (Sigma-Aldrich) according to the manufacturer's instructions for the preparation of RNA from bacteria, with one modification to the protocol. During the DNase I digestion step, the incubation time was extended from 15

minutes to 1.5 hours. RNA concentration and purity was determined using a NanoVue Plus spectrophotometer (GE Healthcare, UK).

2.3.4.2 Reverse transcription of RNA samples (RT-PCR)

An Applied Biosystems High Capacity cDNA Reverse Transcription Kit was used to prepare cDNA from the previously purified RNA samples according to the manufacturer's instructions using random primers. 1 µg of RNA sample was diluted in 10 µl of nuclease free water, and used for a single cDNA reaction. Samples were subsequently stored at -20°C.

2.3.4.3 Quantitative polymerase chain reaction (qPCR)

qPCR was used as tool to compare expression of the *EcaAT* and *Ter* genes when expressed from either plasmid pFRANK or pVICTOR2.0. 16S ribosomal RNA (*rrsA*) from *E. coli* was used as a reference transcript. qPCR primers for target genes were designed using an online tool called Primer3 (<http://bioinfo.ut.ee/primer3/>) under the following constraints:

- Primer size ranges from 18-24 bp
- The amplified region of DNA does not exceed 200 bp
- GC content of 50-60%
- T_m range between 60-63°C
- T_m difference between primers does not exceed 5°C
- Maximum 3' self complementarity score of 1

A 1/10 dilution of the previously isolated cDNA in Milli-Q water (18.2 mΩ) was prepared to give a working stock of template at 5 ng/µl based on the concentration of the original RNA sample. A total reaction volume of 20 µl consisted of: 5 µl of diluted template, 0.25 µM forward/reverse primer (see Methods section 2.3.2.4), 1X LuminoCt® SYBR® Green qPCR ReadyMix™, after which each sample was centrifuged at 2000 rpm for 1 minute before running. qPCR reactions were run in triplicate for each sample using the DNAEngine Peltier Thermal cycler coupled with the Chromo4™ Real-Time PCR Detector (BioRad, UK), and data was analysed by the Opticon 3 thermal cycler software program (BioRad, UK). qPCR conditions were set as specified by the manufacturer's instructions for the qPCR of RNA templates with SYBR® Green pPCR ReadyMix™, specifically an initial denaturation step of 95°C for 20

50

seconds, followed by 40 cycles consisting of a denaturation step of 95°C for 3 seconds, and an annealing/extension step of 60°C for 30 seconds. Following this, a melt curve to confirm the formation of single products during qPCR was performed for each sample, to do this, the temperature was raised from 55°C to 95°C and fluorescence readings were taken at 0.2°C increments.

Cycle threshold (C_t) values were determined for each sample using a method first described by Pfaffl (Pfaffl, 2001) and modified by Abad *et al.*, (2010) to determine fold expression relative to the housekeeping gene. The formula is given as:

$$R = \frac{(E_{target\ gene})^{\Delta C_{t\ target\ (control-sample)}}}{(E_{reference\ gene})^{\Delta C_{t\ reference\ (control-sample)}}$$

In this equation, ‘R’ represents the relative abundance of the target gene to the reference gene, ‘E’ is the efficiency or amplification achieved through 1 cycle by the specific primer pair amplifying the detected region of either the target or reference gene and ΔC_t is calculated as the difference in C_t value between the no template control and the sample.

2.3.4.4 Determination of amplification efficiency for qPCR primer set

To maximize the validity of the results obtained from the RT-qPCR experiments, 5 qPCR primer sets for a single target gene were tested for amplification efficiency, and a single set with optimal efficiency was chosen. A 10 – fold serial dilution of cDNA was carried out to provide a range of 5 template quantities of 0.001 ng, 0.1 ng, 1 ng, 10 ng, and 100 ng per 20 µl reaction. qPCR reactions with the tested primer sets were conducted in triplicate for each condition and the final C_t values were plotted against the logarithm of the original template quantity. The gradient of the linear plot was then used to calculate the efficiency of the primer set according to the following formula:

$$E = 10 \left(-\frac{1}{g} \right) \times 100\%$$

Where ‘E’ is the amplification efficiency (%) and ‘g’ is the gradient of the line. Primer sets that displayed an efficiency between 90-110%, and that only produced single products during PCR – as determined by melt curve analysis and gel electrophoresis of samples – were used in further

qPCR experiments. Table 2 shows the chosen primer sets for each gene examined in this study, and their amplification efficiency.

Table 2. 2 List of primers used for RT-qPCR and their respective amplification efficiency.

Gene	Primer set	Amplification efficiency (%)
<i>AAT</i>	AATF_2	98.6
	AATR_2	
<i>Ter</i>	TerF_4	99.9
	TerR_4	
<i>rrsA</i>	rrsAF_3	99.1
	rrsAR_3	

2.3.5 Gel electrophoresis of DNA

For separation of DNA fragments, a 1% (w/v) agarose gel was used. Agarose gels were set in 1X TAE buffer (0.48% (w/v) Tris base, 0.11% (v/v) acetic acid, 0.2% EDTA, pH 8.0), with 0.5X SYBR® Safe. 7 µl of the GeneRuler™ 1kb DNA molecular weight ladder (Figure 2.2) was loaded in the first well of each agarose gel. 4 µl of 5X DNA loading buffer (30% (v/v) glycerol, and 0.25% (w/v) bromophenol blue) was added to each PCR sample before being loaded onto the gel. Gels were run in a Bio-Rad Wide Mini-Sub cell GT Tank (Bio-Rad, UK) containing 1X TAE buffer at 110V for approximately 30 minutes. Visualization and imaging of each gel was done using a Syngene G:Box ChemiHR system (Syngene, UK) with a short wave UV transilluminator.

GeneRuler 1 kb DNA Ladder

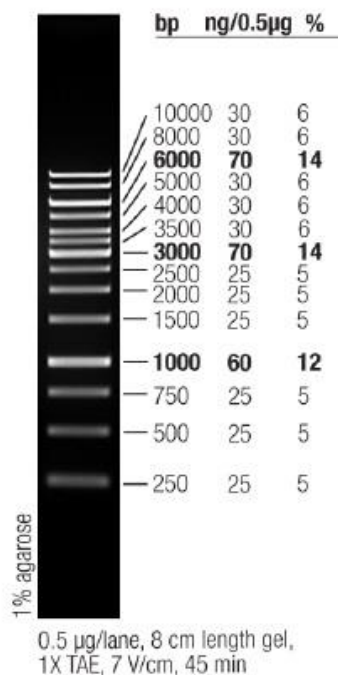


Figure 2. 2 DNA fragment size estimation was done using a GeneRuler™ 1kb DNA Ladder.

2.3.6 Purification of DNA

2.3.6.1 from agarose gel

DNA from agarose gels were visualized via UV light exposure using an UV box (Fotodyne, USA), and bands were excised using a scalpel. A Zymoclean™ Gel DNA Recover Kit (Cambridge Bioscience, UK) was used to purify DNA from the excised gel fragment according to the manufacturer's instructions. DNA was eluted into 6 µl of Milli-Q water (18.2 mΩ).

2.3.6.2 from PCR reaction

DNA from PCR reactions was purified using a DNA Clean & Concentrator kit from Zymo Research (Cambridge Bioscience, UK) according to the manufacturer's instructions. DNA was eluted into 10 µl of Milli-Q water (18.2 mΩ).

2.3.6.3 from *E. coli* culture

Plasmid DNA was purified from 5 mL of *E. coli* culture incubated overnight in LB media. A Qiaprep Spin Miniprep Kit (Qiagen, UK) was used according to the manufacturer's instructions and DNA was eluted into 50 µl of Milli-Q water (18.2 mΩ).

2.3.7 DNA and RNA quantification

DNA and RNA concentration was measured using a NanoVue Plus spectrophotometer (GE Healthcare, UK). 2 µl of DNA sample was loaded and quantified by measuring the absorbance of light at 260 nm. Purity of the DNA/RNA sample was quantified by calculating the ratio of absorbance at 260 nm to 280 nm.

2.3.8 DNA restriction digestion

Restriction enzymes were purchased from either Thermo Fisher Scientific (UK) or New England Biolabs (UK). Restriction digestions were carried out according to the manufacturer's instructions in a total volume of 20 µl for approximately 1 hour at 37°C. Digest reactions were then run on a 1% (w/v) agarose gel for separation of fragments and subsequent gel DNA purification.

2.3.9 DNA ligation

T4 DNA ligase was used for DNA ligations carried out according to the manufacturer's instructions in a total reaction volume of 10 µl. Insert to vector ratios of 3:1 and 5:1 were employed, and reactions were incubated at room temperature overnight before being transformed into chemically competent *E. coli* cells.

2.3.10 Gibson assembly

Gibson assembly was carried out according to Gibson (2009). PCR primers (Appendix) used for amplifying the insert fragment were designed to include a 40 bp linker that shares identity with the regions flanking the site of insertion in the vector. This was done for the primers amplifying from both the 5' and 3' ends of the insert. A master mix containing 320 µl of 5X isothermal reaction buffer (25% PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM MgCl₂, 50 mM DTT, 1 mM

full set of dNTPs, and 5 mM NAD), 0.64 μ l 10 U/ μ l T5 exonuclease (New England Biolabs, UK), 20 μ l 2 U/ μ l Phusion DNA polymerase, 160 μ l 40 U/ μ l Taq DNA Ligase, and Mili-Q water (18.2 m Ω) was made up to a final volume of 15 μ l. To this, 5 μ l of volume containing 100 ng of linearized vector and an equimolar quantity of insert was added and incubated at 50°C for 1 hour. 5 μ l of assembled Gibson mix was then transformed into chemically competent *E. coli* cells.

2.3.11 Transformation into chemically competent *E. coli*

A 50 μ l aliquot of chemically competent BioBlue *E. coli* cells was thawed on ice for approximately 10 minutes. To the aliquot, 5 μ l of the ligation or Gibson assembly mix was added, followed by a further 20 minute incubation on ice. The cells were then heat shocked for 45 seconds at 42°C, and held on ice for 5 minutes before the addition of 1 mL of LB media. Transformations were then recovered at 37°C for 1 hour shaking at 250 rpm. Following this, cells were centrifuged at 4000 rpm for 1 minute and resuspended in 100 μ l of LB media before being plated onto an LB + antibiotic plate and incubated at 37°C overnight.

2.4 Protein methods

2.4.1 SDS-PAGE

2.4.1.1 SDS-PAGE gel composition

SDS-PAGE gels were made in house between glass plates in a casting frame (BioRad, UK) using dH₂O, 30% (w/v) acrylamide mix, 1.5 M Tris-HCl pH 8.8 for resolving gels or 1.0 M Tris-HCl pH 6.8 for stacking gels, SDS, 20% (w/v) ammonium persulfate, and tetramethylethylenediamine (TEMED). The volume of each component used to make the stacking and resolving gel are listed in table 2.3.

Table 2. 3 List of ingredients used to make resolving and stacking SDS-PAGE gels.

	Volume required to make 10 mL total volume (mL)	
	12% resolving gel	Stacking gel
H ₂ O	4.2	7.2
30% (w/v) acrylamide mix	6.4	1.25
1.5 M Tris-HCl pH 8.8	5.2	-
1.0 M Tris-HCl pH 6.8	-	1.25
SDS	0.16	0.1
20% (w/v) Ammonium persulfate	0.024	0.03
TEMED	0.02	0.01

2.4.1.2 Sample preparation and running conditions

1 mL of *E. coli* culture was centrifuged at 14 000 rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 100 µl of buffer A (20 mM Tris pH 8.0, 0.3 M NaCl, and 10 mM imidazole) and sonicated on ice at 12 microns for 10 seconds using a Soniprep 150 plus (MSE, UK). Samples were centrifuged again at 14 000 rpm for 10 minutes and 20 µl of supernatant was removed and mixed with 5X SDS-PAGE loading buffer (10% (w/v) SDS, 20% (v/v) glycerol, 10 mM DTT, 0.2 M Tris pH 6.8, and 0.05% bromophenol blue) to a final concentration of 1X. Samples were then incubated at 98°C for 3 minutes before loading 10 µl onto the SDS-PAGE gel. 6 µl of a Pierce Unstained Protein Molecular Weight Marker (Thermo Fisher) was run in the first well as an indicator for protein size (Figure 2.3). Gels were run in SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS) at 30 mA per gel for approximately 1 hour using a Mini-PROTEAN® Tetra System (BioRad, UK) connected to a PowerPac™ Basic (BioRad, UK).

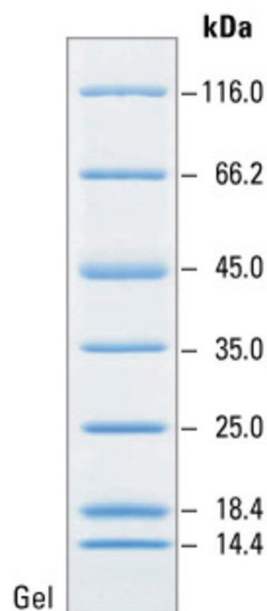


Figure 2. 3 Protein fragment size estimation was done using a Pierce™ Unstained Protein MW Ladder.

2.4.1.3 Staining/Destaining and imaging of SDS-PAGE gel

Following gel electrophoresis, SDS-PAGE gels were stained with Coomassie Blue stain solution (0.2% (w/v) Coomassie blue, 10% (v/v) acetic acid, 50% (v/v) methanol) for 1 hour. Gels were then de-stained in a 10% (v/v) acetic acid and 30% (v/v) methanol solution until protein band could be visualized clearly. SDS-PAGE gels were visualized in a Syngene G:Box ChemiHR and the paired GeneSNAP software (Syngene, UK) using white light.

2.4.2 Recombinant protein expression in *E. coli*

E. coli strain C43 (DE3) heterologously expressing the desired protein was grown at 37°C in LB broth supplemented with the relevant antibiotic, overnight. The culture was then diluted 1/100 into 1L of 2TY + antibiotic and grown at 37°C and 250 rpm until an OD₆₀₀ of 1.0 was reached. At this point gene expression was induced with the addition of either rhamnose (final concentration of 0.2% (w/v)) or IPTG (final concentration of 0.4 mM) – depending on the promotor– and further incubated for another 16 hours at 37°C. Cultures were centrifuged at 4000 rpm for 20 minutes at 4°C and cell pellets was harvested and stored at -80°C for future use.

2.4.3 Protein purification using His-Tag

The cell pellet was removed from -80°C, allowed to thaw on ice, resuspended in 2.5 mL of Buffer A (20 mM Tris pH8.0, 300 mM NaCl, 10 mM imidazole), and sonicated using a Soniprep 150 plus (MSE, UK) on ice at 12 microns for four intervals of 30 seconds. The sample was centrifuged at 14,800 rpm for 15 minutes to obtain the soluble fraction. The soluble fraction obtained was then loaded onto a Poly-Prep chromatography column (0.8 x 4 cm, BioRad, UK) with a pre-equilibrated metal affinity resin charged with cobalt (Clontech, UK). The soluble fraction was run through the column, followed by a column wash with 20 mL of Buffer A. The His-tagged protein was then eluted from the column into a final volume of 2 mL using a mixture of Buffer A and Buffer B (20 mM Tris pH8.0, 300 mM NaCl, 1 M imidazole) that contains a final imidazole concentration of 500 mM.

2.4.4 Crude cell extract preparation

The cell pellet was removed from -80°C, allowed to thaw on ice, resuspended in 5 mL of resuspension buffer (50 mM Tris-HCl pH 7.5, protease inhibitor tablet EDTA-free) and sonicated using a Soniprep 150 plus (MSE, UK) on ice at 12 microns for four intervals of 30 seconds. The sample was then centrifuged at 14,800 rpm for 15 minutes to obtain the soluble fraction. The soluble fraction was further concentrated using a Protein Concentrator PES spin column with a 10K MWCO (Molecular Weight Cut Off) (Pierce, UK) by centrifuging at 14,800 rpm until the final sample concentration was approximately 1 mL.

2.4.5 Protein quantification

Protein concentration was determined using the Bradford Method (Bradford, 1976). Bovine serum albumin (Pierce, USA) was used as a protein standard, and using a 2:7 dilution of Bradford reagent, (BioRad, UK) a standard curve ranging from 0-100 µl/mL was used for sample quantification. Dilutions of protein samples were prepared in dH₂O. Absorbance of standards and samples were read at 595 nm using a Cary50 Bio (Varian, UK).

2.4.7 pH indicator assay of esterase activity

Esterase activity was determined with a spectrophotometric assay using a pH indicator. The final assay solution was prepared by mixing BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic

acid) buffer (5 mM pH 7.2), 1 mM ethyl octanoate, -decanoate, or butyl octanoate, -decanoate as substrate, 4-nitrophenol (4.5 mM dissolved in 5 mM BES pH 7.2; $\epsilon=18 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$) as a pH indicator, 3.9% acetonitrile, and 1 μl purified esterase in a final volume of 1 mL. Reaction rate was determined by following the decrease in absorbance at 404 nm in 96 microtiter plates (Greiner Bio-One, UK) using a BioTek Synergy HT plate reader (BioTek, UK) at 37°C for 10 minutes. Initial rates were calculated from the slope of the linear portion of the curve, usually the first 120 seconds.

2.4.8 p-nitrophenyl acid assay of esterase activity

Esterase activity was determined spectrophotometrically using a Cary 50 Bio spectrophotometer (Varian, UK). Reactions were carried out in a sodium phosphate buffer (50 mM pH 7.5) using either *p*-nitrophenol acetate, -butyrate, -octanoate, or -decanoate (*p*NPA, *p*NPB, *p*NPO, and *p*NPD 10 mM dissolved in DMSO) as substrates. Buffer solution was mixed with increasing volumes of *p*-nitrophenyl substrate and the reaction was started with the addition of 10 μl of protein to a final reaction volume of 1 mL. The amount of liberated *p*-nitrophenol was determined at 400 nm ($\epsilon=15 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and 25°C. From the initial reaction rate, the esterase activity was calculated. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 mmol *p*-nitrophenol/min under assay conditions. Kinetic parameters were calculated using Sigma Plot graphing software (Systat software Inc.).

2.4.9 p-nitrophenyl acid assay of lipase activity

Lipase activity was determined spectrophotometrically using a 6305 UV/Vis spectrophotometer (Jenway, UK). Reactions were carried out in a sodium phosphate buffer (50 mM pH 7.5) using *p*-nitrophenol myristate or -palmitate (10 mM dissolved in hexane). Buffer solution was mixed with either 200 μM or 50 μM of *p*-nitrophenol substrate, 1% (v/v) Triton X-100, and the reaction was started with the addition of 30 μl protein to a final volume of 1 mL. Reaction was incubated at 37°C. The amount of liberated *p*-nitrophenol was determined at 410 nm ($\epsilon=15 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) at various time points up to 3 hours. From the initial reaction rate, the esterase activity was calculated. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 mmol *p*-nitrophenol/min under assay conditions.

2.4.10 Assay for Alcohol Acyltransferase (AAT) activity

Reactions were carried out in a total volume of 500 μ l containing: 10 μ l crude cell lysate of *E. coli* strain C43 (DE3) expressing the AAT enzyme, buffer (50 mM Tris HCl pH 8.5, 10% (v/v) glycerol, and 1 mM DTT), 10 mM butanol, and 0.75 mM hexanoyl-CoA. Reactions were incubated at 30°C for 30 minutes before being halted with the addition of 50 μ l of 10% (w/v) SDS. Each reaction was then extracted into 100 μ l of hexane for future product analysis by GC-MS.

2.4.11 Assay for trans-2-enoyl-CoA reductase (TER) activity

Ter activity was determined on a Cary 50 Bio spectrophotometer (Varian, UK) by measuring the decrease in absorbance at 340 nm. Reactions were carried out in a total volume of 1 mL containing: phosphate buffer pH 6.2, 200 μ M NADH, 2 μ l FAD, 200 μ M crotonyl-CoA, and 5 μ l of crude cell lysate of *E. coli* strain C43 (DE3) expressing the Ter enzyme. All ingredients, except for the cell lysate, were pre-incubated at 30°C for 5 minutes before the addition of the lysate and the commencement of absorbance reading. Initial reaction rates were calculated from the slope of the linear portion of the curve, usually the first 60 seconds.

2.4.12 Malachite Green assay for terpene synthase activity

The malachite green assay was performed in 96-well microtiter plates (Greiner Bio-One, UK) in a total volume of 50 μ l according to Vardakou *et al.*, (2014). The assay mix contained malachite green assay buffer (25 mM MES, 20 mM CAPS, 50 mM Tris, 2.5 mU of inorganic pyrophosphatase (*Saccharomyces cerevisiae*), 5 mM MgCl₂) pH 7.5, 0.008 μ M purified GES enzyme, geranyl pyrophosphate (GPP) ranging in concentration from 10-400 μ M, and a fixed concentration of geraniol (either 0, 100, 200, or 400 μ M). Reactions were set up on ice and incubated at 30°C for 5 minutes. The reaction was halted by the addition of 12 mL of malachite green development solution, and incubated a further 15 minutes at 30°C prior to reading at 623 nm on a BioTek microplate reader. Malachite green development solution was prepared by mixing 10 mL of malachite green dye stock solution (300 mL of 18 M H₂SO₄ mixed with 1.5 L of water and 2.2 g of malachite green powder) with 2.5 mL of 7.5% (w/v) ammonium molybdate, and 0.2 mL 11% (v/v) Tween 20. Standard curves of monophosphate (Pi) and

pyrophosphate (PPi) ranging from 0.39-50 μ M were used for product quantification. Kinetic parameters were calculating using Sigma Plot graphing software (Systat software Inc.).

2.4.13 3D protein structure construction and analysis of EcAAT from *A. chinensis*

2.4.13.1 3D protein structure modelling

A 3D model of EcAAT was built by comparative modelling based on the high-resolution crystal structure of homologous proteins. Swiss-Model (Arnold *et al.*, 2006) was used for selecting 3D models with the closest homology available in the PMDB. The crystal structures of three proteins (PMDB codes: 4KEC, 4G22, and BGH2) were selected as the template for EcAAT model construction, which was then done using MODELLER 9.18 software (www.salilab.org/modeller/). The model showing the lowest MODELLER objective function and RMSD with respect to trace (C α) atoms of the crystal structure templates was carried forward.

2.4.13.2 Binding pocket identification

The solvent pocket associated with the AAT active site motif were identified using the ICMPOCKETFINDER feature of ICM software (Abagyan *et al.*, 1994; www.molsoft.com). This feature identifies all voids and pockets of the protein 3D structure and measures the volume and area of each. As well, it identifies the surrounding residues that compose said pockets. The pocket associated with the acyltransferase catalytic motif (HxxxD) was identified as the substrate binding pocket, and the residues composing this pocket were individually manipulated using ICM software to identify potentially favourable changes in binding pocket size. From this, a selection of mutations were chosen for further *in vivo* analysis of substrate specificity.

2.5 Cell biology methods

2.5.1 Growth curves of wild type *E. coli* grown in the presence of butanol and butyl esters

Wild type cultures of *E. coli* strain DH5 α were incubated in 5 mL cultures of LB media overnight at 37°C and 250 rpm. Following this, cultures were newly inoculated into 7 mL of LB media to a final OD₆₀₀ of 0.4 and incubated at 37°C and 250 rpm with the OD₆₀₀ being recorded every 30 minutes with a 6305 spectrophotometer (Jenway, UK). 1.5 hours post inoculation, varying concentrations of either a butyl ester (butyl acetate, -butyrate, -hexanoate, -octanoate, and -decanoate) or butanol was individually added to the cultures. Cultures continued to be incubated at 37°C and 250 rpm, and the OD₆₀₀ continued to be recorded every 30 minutes until one hour after stationary phase was reached. Each condition was run in triplicate.

2.5.2 Growth curves of wild type *C. acetobutylicum* and *C. saccharoperbutylacetonicum* in the presence of butyl esters

30 mL of RCM media was inoculated with 200 μ l of a glycerol stock culture of either *C. acetobutylicum* or *C. saccharoperbutylacetonicum* and incubated at 32°C overnight in an anaerobic stationary incubator. Following this, cultures were newly inoculated into 15 mL of RCM media with 5% of the overnight culture, and then incubated at 32°C and 150 rpm. After 1.5 hours of incubation, the OD₆₀₀ of each culture was recorded and butyl esters at various final concentrations were added individually into each culture using a Hamilton syringe to pierce the bottle stopper. The cultures were then incubated at 32°C and 150rpm with OD₆₀₀ readings being taken every 1.5 hours for approximately 8 hours. Samples were run in duplicate.

2.5.3 Butyl ester feeding in *E. coli* cultures heterologously expressing an esterase from *Pseudomonas fluorescens*

E. coli strain DH5 α expressing the plasmid pJOE2792::PFE (*Pseudomonas fluorescens* esterase) was incubated in LB media supplemented with ampicillin (100 μ g/mL) at 37°C and 250 rpm for

16 hours. The cultures were then diluted 1:100 and grown to an OD₆₀₀ of 0.4 in LB with the relevant antibiotic before being induced with the addition of rhamnose (0.2% w/v). Following this, cultures were further incubated another 2 hours under the same culture conditions before the addition of either 10 mM butyl octanoate or -decanoate. Wild type DH5α *E. coli* cultures individually supplemented with 10 mM of either butyl ester were used as controls. Cultures were then further incubated at 37°C and 250 rpm for a total of 48 hours. Following this, 5 mL samples of each culture was taken at 12, 24, and 48 hours and the OD₆₀₀ was determined. Each sample was then centrifuged at 4000 rpm for 5 minutes and the supernatant poured into a separate tube and stored at -20°C for future analysis.

2.5.4 Analysis of supernatant esterase activity due to cell lysis in *E. coli*

E. coli strain DH5α expressing the plasmid pJOE2792::PFE (*Pseudomonas fluorescens* esterase) was incubated in LB media supplemented with ampicillin (100 µg/mL) at 37°C and 250 rpm for 16 hours. The cultures were then diluted 1:100 and grown to an OD₆₀₀ of 0.4 in LB with the relevant antibiotic before being induced with the addition of rhamnose (0.2% w/v). Following this, cultures were incubated at 37°C and 250 rpm for 12 hours before being divided into two groups. Group one had 10 mM of either butyl octanoate or -decanoate added to the culture and was further incubated another 12 hours at 37°C and 250 rpm, while the cultures of group two were centrifuged at 4000 rpm to separate culture supernatant and pellet and the supernatant exclusively was incubated with 10 mM of either butyl octanoate or -decanoate for 12 hours at 37°C and 250 rpm. Following this, 5 mL samples from the cultures of each group were centrifuged at 4000 rpm for 5 minutes and the supernatant was poured into a separate tube and stored at -20°C for future analysis.

2.5.5 1-Butanol production by *E. coli*

E. coli strain JCL166 expressing the relevant plasmids for butanol production was incubated in LB media supplemented with appropriate antibiotic (ampicillin 100 µg/mL, kanamycin 50 µg/mL, hygromycin 100 µg/mL, tetracyclin 20 µg/mL) at 37°C and 250 rpm for 16 hours. Cultures were then diluted 1:100 into 25 mL of TB-2% (w/v) glucose media with relevant antibiotics in anaerobic vials (Supelco, UK), and grown to an OD₆₀₀ of 0.8 aerobically before induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following this, cultures were grown aerobically for a further 2 hours before the displacement of oxygen from cultures

vials using an N₂ stream, and the capping of vials with an anaerobic crimp cap (Supelco, UK). Cultures were then incubated at 37°C and 250 rpm with 5 mL samples taken at 24 hour intervals for 96 hours. As well, cultures were supplemented with 1.5% glucose daily. The OD₆₀₀ of samples were determined and stored at -20°C for future analysis.

2.5.6 Shake-flask aerobic production of butyl esters in *E. coli* with substrate feeding

E. coli strain C43 (DE3) expressing a relevant butyl ester producing plasmid was incubated in LB media supplemented with relevant antibiotic (ampicillin 100 µg/mL, or hygromycin 100 µg/mL) at 37°C and 250 rpm for 16 hours. Cultures were then diluted 1:100 and grown to an OD₆₀₀ of 0.8 in TB + 2% (w/v) glucose media with relevant antibiotics before being induced with 0.4 mM IPTG. Following this, cultures were further incubated 2 hours at 20°C and 250 rpm before the addition of 5 mM octanoic acid and 10 mM butanol. A culture of wild type C43 (DE3) was used as a negative control. After 18 hours of incubation at 20°C and 250 rpm the OD₆₀₀ of each sample was determined and culture pellet and supernatant were separated and stored at -20°C for future analysis.

2.5.7 Shake-flask anaerobic production of butyl esters in *E. coli* with substrate feeding

E. coli strain JCL166 expressing the relevant plasmids for butanol and butyl ester production was incubated in LB media supplemented with appropriate antibiotic (ampicillin 100 µg/mL, kanamycin 50 µg/mL, hygromycin 100 µg/mL, tetracycline 20 µg/mL) at 37°C and 250 rpm for 16 hours. Following this, cultures were grown as described in section 2.5.4 with several modifications, including: cultures were induced with 0.4 mM IPTG as opposed to 0.1 mM, after induction cultures were grown at 30°C as opposed to 37°C, and 24 hours after the anaerobic switch cultures were supplemented with 3 mM octanoic acid. A sample was then taken 48 hours post-anaerobic switch to determine the OD₆₀₀ and the culture supernatant and pellet were separated and stored at -20°C for future analysis.

2.5.8 Shake-flask culturing of *E. coli* for fatty acid profile analysis

E. coli strains C43 (DE3) and JW5020 ($\Delta FadE$) were incubated in LB media supplemented with relevant antibiotics (kanamycin 50 $\mu\text{g/mL}$) at 37°C and 250 rpm for 16 hours. Cultures were then diluted 1:100 and grown to an OD₆₀₀ of 0.8 in M9 + 2% (v/v) glucose media with relevant antibiotics, and then incubated at 20°C before the exogenous addition of 2.5 mM octanoic acid. Following this, cultures were incubated at 20°C and 250 rpm for 18 hours before samples were taken to determine OD₆₀₀, and culture pellets were stored at -20°C for future analysis.

2.5.9 Heterologous expression of thioesterase in *E. coli*

E. coli strains C43 (DE3) and JW5020 ($\Delta FadE$) expressing a plasmid holding a thioesterase (TE) named *FATB1* from *Cuphea palustris* were incubated in LB media supplemented with relevant antibiotics (ampicillin 100 $\mu\text{g/mL}$ and/or kanamycin 50 $\mu\text{g/mL}$) at 37°C and 250 rpm for 16 hours. Cultures were then diluted 1:100 and grown to an OD₆₀₀ of 0.8 in M9 + 1% (v/v) glycerol media with relevant antibiotics before being induced with 0.4 mM IPTG. At this time, some cultures were additionally supplemented with 4 $\mu\text{g/mL}$ of the antibiotic cerulenin for fatty acid elongation retardation. Following induction, all cultures were incubated at 30°C and 250 rpm for 24 hours before samples were taken to determine OD₆₀₀, and culture pellets were stored at -20°C for future analysis.

2.5.10 Shake flask production of geranyl acetate in *E. coli* with substrate feeding

E. coli harbouring the plasmid holding the AAT from *Rosa hybrida* was inoculated into LB media supplemented with kanamycin (50 $\mu\text{g/mL}$) and grown overnight at 37°C and 250 rpm. Cultures were then inoculated at 1:100 into 25 mL of TB + 2% (w/v) glucose media with relevant antibiotic with kanamycin (50 $\mu\text{g/mL}$), and grown at 37°C until an OD₆₀₀ of 1 was reached. Cultures were then induced with 0.4 mM IPTG and moved to 20°C and 250 rpm for 2 hours before being fed 10 mM acetic acid and 0.5 mM geraniol. Cultures were then further incubated for 18 hours at 20°C and 250 rpm. Following this, the OD₆₀₀ was measured and cultures samples of both the supernatant and pellet were taken and stored at -20°C for future analysis.

2.5.11 Shake-flask geraniol and geranyl acetate production in *E. coli*

Strains DLGA2 and DLGA3 were inoculated into LB media and grown overnight at 37°C. Cultures were then inoculated at 1:100 into 25mL of TB + 2% (w/v) glucose media with relevant antibiotic, and grown at 37°C until an OD₆₀₀ of 1 was reached.

2.5.11.1 Geraniol

Cultures of the DLG2 strain were then induced with 50 µM IPTG, and a 10% (v/v) dodecane top layer was added to trap geraniol and other monoterpenes. Cultures were then transferred to a rotary shaker (250 rpm) at 30°C. After 24 hours, the OD₆₀₀ in the aqueous phase was measured and a sample of the dodecane layer was taken and diluted into ethyl acetate for analysis by gas-chromatography mass-spectrometry (GC-MS).

2.5.11.2 Geranyl acetate

Cultures of the DLGA3 strain were induced with 100 µM IPTG and supplemented with either 0, 5, 10 or 20 mM acetic acid before a 10% (v/v) dodecane top layer was added to trap geranyl acetate. Cultures were then incubated at 30°C in a rotary shaker (250 rpm) for 24 hours and analysed as described above.

2.5.12 Analysis of endogenous isomerization and reduction of geraniol and nerol in *E. coli*

E. coli strain C34 (DE3) was inoculated into LB media and grown overnight at 37°C. The following day, cultures were inoculated at 1:100 into 5 mL of TB + 2% (w/v) glucose media, and grown at 37°C in a rotary shaker (250 rpm) until an OD₆₀₀ of 4 was reached. Cultures were then fed either 0.5 mM geraniol or 0.5 mM nerol and further incubated at 37°C and 250 rpm for 6 hours before supernatant samples were taken and stored at -20°C for future analysis.

2.5.13 Fed-Batch fermentation of Geraniol and Geranyl Acetate in Bioreactor

2.5.13.1 MTB media

Strains DLG2 and DLGA3 were incubated overnight at 37 °C in 100 mL of LB media and used to inoculate a 1.5 L fermenter (BIOSTAT B plus MO5L, Sartorius, Germany) containing 1.2 L of MTB media containing the relevant antibiotics. Strain DLG2 was induced when OD₆₀₀ reached approximately 20 with 50 µM IPTG, and a 10% (v/v) dodecane top layer was then fed into the culture. Strain DLGA3 was induced when OD₆₀₀ reached approximately 20 with 125 µM IPTG, 20 mM acetic acid and a 10% (v/v) dodecane top layer were then fed into the culture. Culture temperature was maintained at 30°C and pH was maintained at 6.8 by automatic addition of 5 M KOH or 5 M H₂SO₄. Antifoam 204 was used to minimize foam development. Dissolved oxygen was maintained at 20% saturation through air flow and stir speed. Intermittent feeding of a MTB + 65% (w/v) glucose solution was initiated to maintain culture glucose concentration between 5-10 g/L. Fermentation samples were periodically collected to determine culture OD₆₀₀, glucose concentration, and terpene product formation.

2.5.13.2 FM media

Strain DLGA3 was also run in fed-batch fermentation using a semi-defined fermentation media (FM). Conditions were as described above for strain DLGA3, with an exception being that the culture was induced with 100 µM IPTG at an OD₆₀₀ of 5.5. Intermittent feeding of an FM + 65% (w/v) glucose solution was used to maintain culture glucose concentration between 5-10 g/L.

2.5.14 Solubility of geraniol and geranyl acetate

10 mL of TB+2% (w/v) glucose media was supplemented with either 100 mg/L of geraniol or geranyl acetate followed by the addition of a 10% (v/v) dodecane top layer before being incubated at 30°C and 250 rpm for 6 hours. After incubation, a sample of the organic dodecane top layer was taken directly for analysis by GC-MS, while the aqueous layer was further extracted with hexane before analysis.

2.6 Lipid product extraction

2.6.1 Ester extraction

2.6.1.1 From culture supernatant

25 mL of culture was removed and centrifuged at 20°C and 14,800 rpm for 10 minutes to separate the culture pellet and supernatant. The supernatant was poured off into a glass vial and extracted with 1.5 mL of hexane by vortexing in three 30 second intervals. The supernatant sample was then centrifuged again at 14, 800 rpm for 5 minutes to separate the organic and aqueous phases and ~300 µl of the organic phase was removed and placed in a 1.5 mL gas chromatography vial (Varian, UK) to run on the GC-MS.

2.6.1.2 From culture pellet

The remaining pellet was resuspended in 3 mL of 0.9% (w/v) NaCl and sonicated on ice at 12 microns for four intervals of 30 seconds. Pellet samples were then extracted with 500 µl of hexane and vortexed in three 30 second intervals. Samples were then centrifuged at 14, 800 rpm for 5 minutes and ~300 µl of the organic phase was placed in a 1.5 mL gas chromatography vial (Varian, UK) to run on the GC-MS.

2.6.2 Free fatty acid esterification and extraction

2 mL of culture was centrifuged at 5000 rpm for 10 minutes to separate the supernatant and pellet, the supernatant was discarded. To the pellet, 10 µg of undecanoic acid (C:11) was added as an internal standard, and then the pellets were resuspended in 3 mL of 1 M MeOH-HCl before being heated to 80°C for 90 minutes. Samples were then cooled to room temperature before the addition of 1 mL of 0.9% (w/v) NaCl followed by extraction with 500 µl of hexane. and ~300 µl of the organic phase was placed in a 1.5 mL gas chromatography vial (Varian, UK) to run on the GC-MS.

2.7 Product quantification methods

2.7.1 Gas Chromatography-Mass Spectroscopy (GC-MS)

2.7.1.1 For butanol and esterified acid products

Butanol and butyl ester products were quantified by a model 7890B gas chromatograph and 5977A mass spectrometer (Agilent technologies, Stockport, UK). Samples were separated on a DB-FFAP 30m x 20 μ m x 0.25 μ m capillary column under the following conditions: 1 μ l of sample was injected onto the column which was held at 40°C for 5 minutes, the temperature was then ramped at a gradient of 15°C/min to a final temperature of 250°C and held for 4 minutes. Butanol and butyl ester products typically eluted between 4 and 12 minutes and were monitored on both MS and FID detectors. The concentration of product was quantified using calibration curves for each compound analysed.

2.7.1.2 For terpene and terpene ester products

Terpene and ester products were quantified by a model 7890B gas chromatograph and 5977A mass spectrometer (Agilent technologies, Stockport, UK). Samples were separated on a DB-FFAP 30m x 20 μ m x 0.25 μ m capillary column under the following conditions: 1 μ l of sample was injected onto the column which was held at 40°C for 1 minute, the temperature was then ramped at a gradient of 20°C/min to a final temperature of 250°C and held for 8 minutes. Terpene and ester products typically eluted between 7 and 11 minutes and were monitored on both MS and FID detectors. The concentration of product was quantified using calibration curves for each compound analysed.

2.7.2 Ion Chromatography (IC)

During fermentation, culture glucose was monitored using ion chromatography with a Dionex 5000+ fitted with a 4x250 mm analytical CarboPac PA1 column (Thermo Fisher, UK). Filtered supernatant was injected and run isocratically for 15 minutes at a flow rate of 1.0 mL/min (50 mM NaOH at 30°C). Glucose concentration was quantified using a calibration curve.

3. Validation of an esterification strategy for the detoxification of butanol production in *E. coli*

3.1 Introduction

Butanol is a valuable compound and intermediate in a number of industries, and its applications include use as a solvent, as a feedstock chemical in the plastics industry, as an extractant in the food, flavour, cosmetic and pharmaceutical industries, and as a renewable fuel (Parekh *et al.*, 1999; Dürre, 2007; Ezeji *et al.*, 2007; Papoutsakis, 2008; Lee *et al.*, 2008; Lütke-Eversloh and Bahl, 2011; Ndaba *et al.*, 2015). With all of these commercial applications, the global demand for n-butanol is estimated to be more than 5 million tons per year, with a market value of nearly 6 billion USD for butanol alone, and 50 billion USD for butanol derivatives (Branduardi, 2014; www.nexant.com). This ever-growing demand, paired with the fluctuating cost of the petroleum-based feedstocks used to chemically synthesise butanol, has stimulated interest in the development of renewable and sustainable strategies for butanol production (Zheng *et al.*, 2009; Nielsen *et al.*, 2009). Specifically, microbial platforms for butanol production have been a growing market in the biotechnology industry over the last few decades. Butanol can be produced naturally by carbohydrate fermentation using certain species of clostridia via the ABE fermentation pathway (Introduction, Figure 1.1), with wild type solventogenic strains producing between 12-13 g/L from simple sugars in batch fermentation (Papoutsakis, 2008). To improve upon these titres to an economically practical level, a significant amount of research has gone into not only optimizing butanol production in clostridia, but also heterologous reconstruction of the butanol biosynthetic pathway in other more tractable microbial hosts that possess more established genetic toolboxes. This heterologous expression in non-native strains has been done in a number of organisms including: *E. coli*, *S. cerevisiae*, *P. putida*, *B. subtilis*, and cyanobacteria (Nielsen *et al.*, 2009; Shen *et al.*, 2011; Lan *et al.*, 2011; Schadeweg *et al.*, 2016). However, the butanol titres achieved by these heterologous hosts have been moderate, and have not yet exceeded those of the natural clostridial producers, even with extensive metabolic engineering of both the butanol pathway and the host strain (Atsumi *et al.*, 2008; Lee *et al.*, 2011; Shen *et al.*, 2011; Chin *et al.*, 2013; Wang *et al.*, 2015; Dong *et al.*, 2016).

A significant impediment to achieving higher microbial butanol titres, not only in the heterologous hosts – but also in the native clostridial species – is the high cytotoxicity of butanol. Butanol exhibits inhibitory effects on microbial growth at low concentrations, with even native clostridial producers only able to tolerate 2% (v/v) (Papoutsakis, 2008; Liu *et al.*, 2012). Non-native producers are generally less tolerant to butanol, with *E. coli* strains rarely being able to tolerate above 1.5% (v/v) (Fischer *et al.*, 2008; Knoshaug *et al.*, 2009). The modes of toxicity butanol exert on cellular function are multifaceted and often intimately linked to general stress responses, making them difficult to address (Rutherford *et al.*, 2010; Dunlop *et al.*, 2011). Butanol is relatively hydrophobic and accumulates in cytoplasmic membranes where it disrupts structure and function by increasing membrane fluidity. This has several trickle-down effects, including the inability to maintain transmembrane ion gradients, leading to energetic uncoupling and leakage of intracellular metabolites. Additionally, it has been implicated in increasing the production of reactive oxygen species (ROS), and hindering metabolite transport and biosynthesis (Isken and de Bont, 1998; Dong *et al.*, 2009; Nielsen *et al.*, 2009; Rutherford *et al.*, 2010). To address this microbial toxicity, a number of groups have employed rational (directed) and/or random (combinatorial) approaches to developing butanol-resistant *E. coli* strains. Several notable successes at improving tolerance include the co-overexpression of multiple molecular chaperones, expression of a heterologous efflux pump from *P. putida*/directed evolution of a native efflux pump, expression of metallothioneins to act as scavengers for ROS, and global transcription machinery engineering (Zingaro *et al.*, 2012; Chin *et al.*, 2013; Fisher *et al.*, 2014; Bui *et al.*, 2015; Zhang *et al.*, 2015). Additionally, butanol recovery techniques have been developed that are designed to minimize the fermentation culture's exposure to butanol. Strategies such as gas-stripping and *in situ* liquid-liquid extraction have successfully been implemented, usually in complementation with strain engineering (Ezeji *et al.*, 2007; Fischer *et al.*, 2008; Zheng *et al.*, 2009). However, butanol tolerance remains a complex, multigenic phenotype, and the successes that have been achieved have been moderate. Ultimately, there has been no all-encompassing solution to the problem of microbial butanol toxicity through strain engineering thus far.

An alternative strategy to combat microbial toxicity may be to modify the butanol molecule itself to be less toxic, as opposed to engineering the bacterial system for increased tolerance. Similar to the effects of extraction this would sequester the butanol into a less reactive form that does not accumulate in – and disrupt the function of – cell membranes, thus improving the achievable titres in culture. A simple molecule into which to sequester butanol would be an

ester. Previous work done to engineer *E. coli* strains for the production of fatty acid methyl and ethyl esters (FAMEs and FAEEs, respectively) have reported their accumulation has no impact on culture health – suggesting the same may be the case for butyl esters (Elbahloul *et al.*, 2010; Fan *et al.*, 2013; Sherkhanov *et al.*, 2016). An advantage of this strategy over the iterative engineering of *E. coli* for improved butanol tolerance is that *in vivo* butanol esterification requires only a single enzymatic step for the condensation of butanol and an acyl CoA (Figure 3.1).

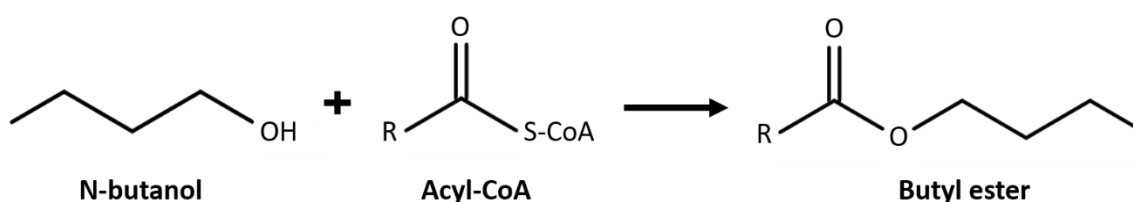


Figure 3. 1 Mechanism showing the *in vivo* enzyme catalysed condensation of butanol and an acyl-CoA to form a butyl ester.

This strategy for addressing butanol toxicity via esterification has not been attempted previously, and as such requires fundamental validation of its potential efficacy. The primary consideration for this strategy is the target ester into which butanol will be sequestered. This ester should possess certain characteristics, including: the ability to accumulate to higher concentrations in culture than butanol with little to no impact on culture health, and the ability to permeate across the plasma membrane into the extracellular milieu. This latter consideration, regarding molecule permeability, is an important consideration with regard to downstream recovery of the butyl ester. Generally, industrial fermentation of *E. coli* strains engineered for the production of long chain FAMEs and FAEEs suffers from high extraction costs as these compounds remain intracellular and therefore require a more intensive recovery process than compounds that are secreted in to the culture supernatant (Elbahloul *et al.*, 2010).

Here, the work carried out was toward investigating butyl esters as a less toxic derivative of butanol in *E. coli* as a potential detoxification strategy. To do this (i) the microbial toxicity of a number of butyl esters of varying acid moiety chain length compared to butanol was assessed,

and (ii) the ability of those butyl esters with minimal microbial toxicity to move across the plasma membrane in *E. coli* was assessed.

3.2 Results and discussion

3.2.1 Identification of an end-product butyl ester with low toxicity to *E. coli*

To determine which butyl ester(s) would be the most suitable candidate for sequestering butanol wild type *E. coli* DH5 α was cultured with exogenously fed butyl esters of increasing acid moiety length from acetate to decanoate, over a concentration range of 0 mM to 100 mM. Cultures fed butanol at the same concentrations were run in parallel for comparison of toxicity (Figure 3.2). The general trend observed from these toxicity tests is that butyl ester toxicity becomes progressively greater as the length of the acid moiety of the ester increases from acetate (C2:0) to hexanoate (C6:0), with the minimum concentration of ester required to impart an observable impact on growth rate decreasing over this range (Figure 3.2). However, it appears that both butyl octanoate and -decanoate exposure had very little impact on the growth rate of *E. coli*; with cultures exposed to even 100 mM of either butyl ester growing comparably to the wild type controls. The observed high toxicity of the shorter chain butyl esters correlates to some degree with the solubility of these compounds, and their resultant bioavailability to the cell. Their solvent-like nature allows them to partition into the hydrophobic cell membrane. An indirect metric to assess the ability of a compound to interact with a membrane is its partition coefficient ($\log P$), which is defined as the ratio of the concentration of a compound between an aqueous (water) and hydrophobic (1-octanol) phase (de Bont, 1998). Compounds with a $\log P$ in the range of 1-3.8 tend to be toxic to the growth of *E. coli* due to this membrane perturbation, while solvents with a $\log P > 4$ being highly insoluble in the media, minimizing bioexposure, and solvents with a $\log P < 1$ typically being water soluble, meaning they would impose any toxic effect via a different mechanism to the solvent-like compounds (Vermuë *et al.*, 1993; Isken *et al.*, 1999; Rutherford *et al.*, 2010; Mukhopadhyay *et al.*, 2015). Table 3.1 shows the $\log P$ value of each of the butyl esters studied in this work. From here we can see that butyl acetate, -

butyrate, and -hexanoate have values within the range of 1-3.8, while butyl octanoate and -decanoate have values above this threshold. Thus, the shorter chain butyl esters were discarded as viable candidates for this strategy, as their toxicity was even higher than butanol, and progressed with C8 and C10 butyl esters as potential end-point products.

Table 3. 1 The 1-octanol/water partition coefficient (Log P) values for the butyl esters evaluated in this work.

Alcohol/Ester	LogP value ^a
Butyl acetate	1.82
Butyl butyrate	2.83
Butyl hexanoate	3.84
Butyl octanoate	4.73
Butyl decanoate	5.88

^a values were determined using the ALOGPS calculator from <http://www.vcclab.org/lab/alogps/>

At a nominal concentration of 100 mM in culture, the aqueous solubility of both butyl octanoate and -decanoate is greatly exceeded (aqueous solubility of butyl octanoate = 3.5 mg/L at 25°C and butyl decanoate = 0.36 mg/L at 25°C; <http://www.thegoodscentscompany.com>), suggesting these compounds truly lack any molecular toxicity. However, an alternative type of microbial toxicity named ‘phase toxicity’ can occur when a culture is grown in the presence of a second organic phase (Bar *et al.* 1987). Occasionally the presence of an aqueous/solvent interface can lead to interfacial cell contact and adsorption, extraction of membrane components, partitioning of nutrients from the aqueous phase, and limited nutrient availability to the cell (Bar *et al.*, 1987; Bothun *et al.*, 2005). To determine whether or not butyl octanoate or -decanoate causes phase toxicity, the concentration of each exogenously fed ester was increased to 125 mM, 150 mM, and 200 mM in culture (Figure 3.3). Even at the highest concentration of 200 mM, these butyl esters

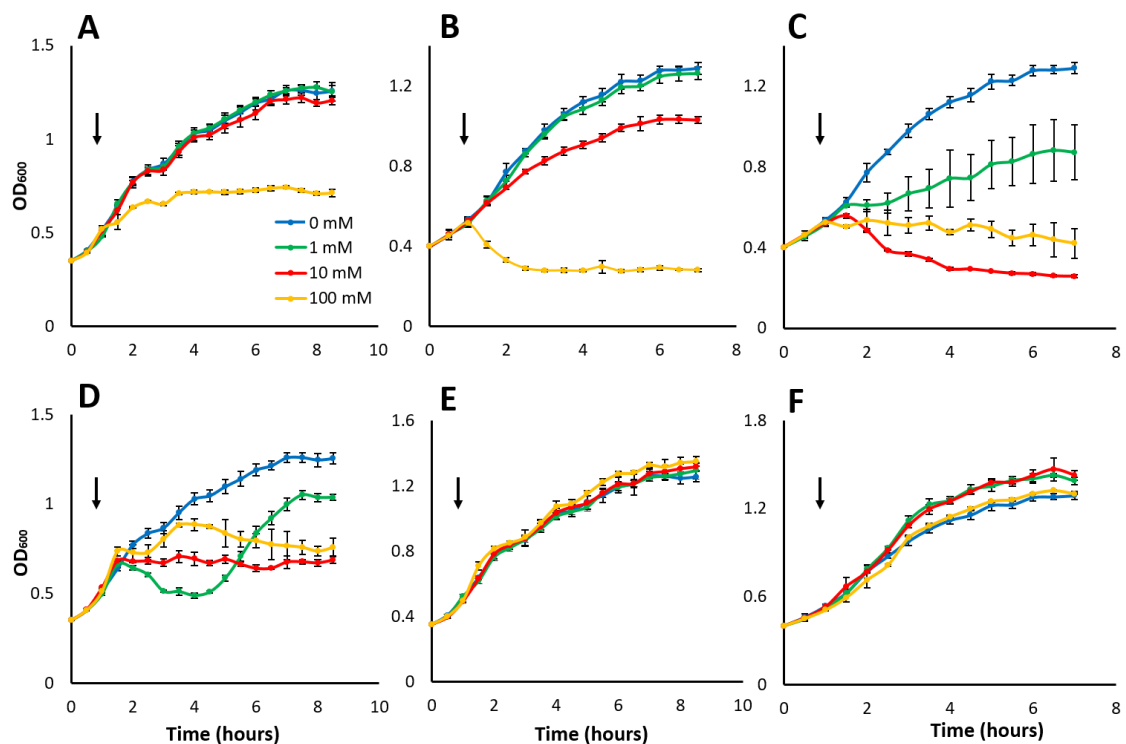


Figure 3. 2 Growth of wild type *E. coli* DH5α fed 0 mM, 1 mM, 10 mM, and 100 mM of either: **A**, butanol; **B**, butyl acetate; **C**, butyl butyrate; **D**, butyl hexanoate; **E**, butyl octanoate; or **F**, butyl decanoate. Cultures were grown at 37°C and 250 rpm, and the OD₆₀₀ was taken every 30 minutes until stationary phase was reached. Butyl ester was added 1 hour after first reading (indicated with the black arrow). Data are the mean ± standard deviation from three biological replicates.

had no impact on the growth rate of *E. coli*, suggesting that they cause no phase toxicity, at least not at these concentrations. Conversely, a concentration of 125 mM butanol resulted in arrestment of culture growth (Figure 3.3). This is unsurprising as this concentration is the equivalent of 1.15% (v/v), which exceeds the reported tolerated level in *E. coli* (Fischer *et al.*, 2008; Knoshaug *et al.*, 2009).

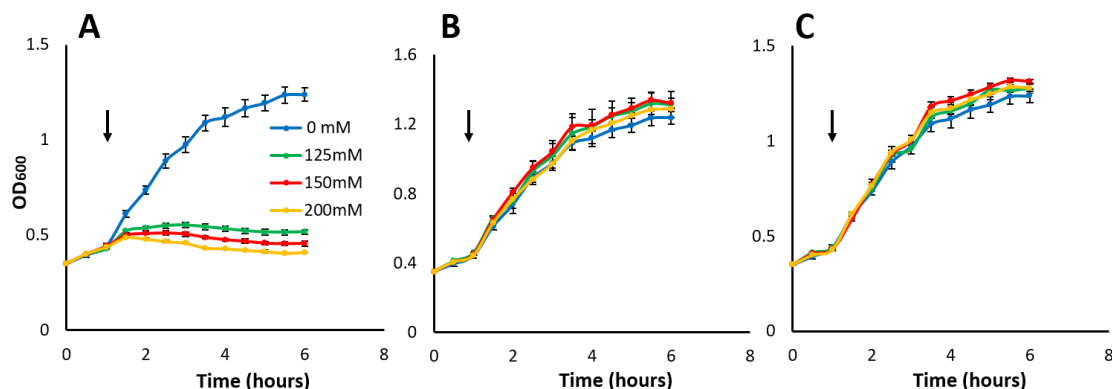


Figure 3.3 Growth rate of wild type DH5α *E. coli* endogenously fed 0 mM, 125 mM, 150 mM, and 200 mM of either: **A**, butanol; **B**, butyl octanoate, or **C**, butyl decanoate. Cultures were grown at 37°C and 250 rpm, and the OD₆₀₀ was taken every 30 minutes until stationary phase was reached. Butyl ester was fed 1 hour after first reading (indicated with black arrow). Data are the mean \pm standard deviation from three biological replicates.

These results suggest that both butyl octanoate and -decanoate could be ideal esters into which to sequester butanol as neither have any impact on *E. coli* culture health at high nominal concentrations, that far surpass the toxicity limit of butanol. However, although they are probably not partitioning significantly into cell membranes, a possible alternative explanation for the lack of an observable toxicity could be that, due to their size, they are too bulky to readily permeate across the cellular membranes into the cell interior to interact with, and disrupt the function of, intracellular components. This would present two significant problems; first, the possibility that these two esters would be more toxic to the cell if produced endogenously, as their inability to cross the plasma membrane may skew the true measure of their toxicity. Second, the inability of these esters to permeate across the plasma membrane into the cell upon external exposure would suggest that they would be equally unable to pass across the membrane out of the cell into the culture media when being made endogenously. Should this be the case, it would make downstream recovery of the butyl ester a more intensive and costly task.

3.2.2 Investigation of the ability of butyl octanoate and decanoate to permeate across the plasma membrane of *E. coli* to the extracellular milieu

To determine whether butyl octanoate and -decanoate are able to move across the plasma membrane into and out of the cell, a non-secreted esterase from *Pseudomonas fluorescens* was heterologously expressed in *E. coli*. Esterases are a class of hydrolase enzyme that are responsible for the hydrolysis of short and medium chain carboxylic esters (Jensen *et al.*, 2016). The rationale behind this strategy was that exogenously fed butyl esters which can move across the *E. coli* cell membranes into the cytoplasm would be hydrolysed by the *P. fluorescens* esterase (PFE) into their alcohol and acid components, which can then be detected by chromatography. Heterologous expression of a non-native esterase was required for this experimental strategy as *E. coli* has no documented esterase enzymes with specific activity for hydrolysing butyl esters. These compounds are not natural products of *E. coli* metabolism and, generally, *E. coli* expresses only low levels of nonspecific esterase activity. While individual esterases have been identified in *E. coli*, such as one from the hormone-sensitive lipase (HSL) family of proteins that has been characterised for hydrolysing carboxylic esters with acyl chains \leq C8, the *in vivo* activity of these esterases is basal (Kanaya *et al.*, 1998; Antonczak *et al.*, 2009). The PFE that was heterologously expressed has been well characterised by Krebsfänger *et al.*, (1998), and shown to be active in hydrolysing ethyl esters. Typically, the substrate preference of an esterase is dictated by either the acyl chain length or the alcohol chain length, but rarely both (Bornscheuer, 2002). This particular esterase from *Pseudomonas* has substrate specificity that is dictated by the acyl chain length of the ester, and is thus promiscuous with regard to the alcohol length it will accept as a substrate. This suggests that it would also be able to hydrolyse butyl esters, such as butyl octanoate and -decanoate, in addition to the ethyl esters it was already characterised for.

To confirm that PFE does in fact have activity for hydrolysing butyl octanoate and -decanoate the *PFE* gene was overexpressed from the rhamnose inducible pJOE2792::*PFE*+His6x plasmid (a generous gift from Dr. Uwe Bornscheuer from the Institut für Technische Biochemie, Universität Stuttgart) in *E. coli* and purified the esterase (Figure 3.4). Activity of this enzyme towards the ethyl and butyl esters of octanoate and decanoate was determined using a continuous *in vitro* pH indicator assay where 4-nitrophenol was used as the indicator. This assay progresses as the given ester is hydrolysed to its alcohol and acid components, which

accumulate in the reaction mixture and increase the local pH. In turn, the 4-nitrophenolate indicator is protonated and the resulting 4-nitrophenol can be quantified at a wavelength of 404 nm (Janes *et al.*, 1998). From the results (Table 3.2), it is evident that PFE does have activity for both ethyl and butyl esters of octanoate and decanoate. It was lower for the butyl esters compared to their ethyl counterparts, which was expected as it had also previously shown lower activity towards methyl esters compared to ethyl esters, suggesting ethyl esters are its preferred substrate (Krebsfänger *et al.*, 1998). For further analysis of PFE enzyme kinetics towards the hydrolysis of esters with C8 and C10 acyl chain lengths, a continuous *in vitro* assay which followed the PFE mediated hydrolysis of either *p*-nitrophenyl octanoate or -decanoate was carried out by quantifying the amount of released *p*-nitrophenol at a wavelength of 410 nm. This later kinetic data confirms the preference of the PFE for the shorter octanoate chain length over decanoate, with k_{cat}/K_m values of $39.9 \mu\text{M}^{-1}\text{s}^{-1}$ and 1.4 , respectively. Ultimately, the PFE enzyme suited our needs as it is a non-secreted esterase, known to be actively expressed in *E. coli*, that possesses activity for hydrolysing medium chain butyl esters.

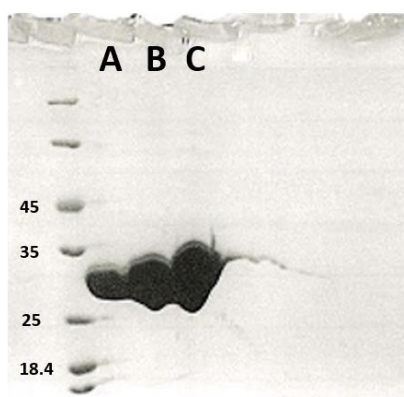


Figure 3. 4 Coomassie-stained SDS-PAGE gel showing the PFE protein purified from a 1 L culture of *E. coli* strain BL21 (DE3) expressing plasmid pJOE2792::PFE+His6x. PFE was purified using a cobalt affinity column and is predicted to be 30 kDa in size. Lane A, B, and C represent 1 μl , 2 μl , and 5 μl , respectively, of a 7 mg/L sample of purified PFE. The size of the bands (in kDa) is indicated to the left of the ladder.

Table 3. 2 *In vitro* activity of PFE for hydrolysing ethyl and butyl octanoate and -decanoate using a pH indicator assay. Activity is mean \pm standard deviation from four replicates.

Substrate	Activity (mmol/min/mg) ^a
Ethyl octanoate	1.48 \pm 0.13
Ethyl decanoate	0.42 \pm 5.7x10 ⁻²
Butyl octanoate	0.42 \pm 7.3x10 ⁻²
Butyl decanoate	0.13 \pm 1.3x10 ⁻²

^a Activities were determined at 37°C and pH 7.2 using purified PFE and 1mM of respective substrate

Table 3.3 *In vitro* activity of PFE for hydrolysing *p*-nitrophenyl octanoate and -decanoate. Kinetic parameters were determined using a colorimetric assay. Data are the mean \pm standard deviation from three replicates.

	<i>p</i> -nitrophenyl octanoate (C8)	<i>p</i> -nitrophenyl decanoate (C10)
K_m (μM)	0.1 \pm 8e ⁻³	23 \pm 4
V_{max} (μmol/mg/min)	8 \pm 0.3	63 \pm 6
k_{cat}/K_m (μM⁻¹/s⁻¹)	39.9	1.4

With confirmation that PFE was able to hydrolyse butyl octanoate and -decanoate esters, we then expressed this enzyme in *E. coli* strain DH5α and fed either 10 mM of octanoate or decanoate butyl esters to growing cultures. The culture supernatant was then analysed at 12, 24, and 48 hours for the presence of ester breakdown products, i.e. butanol and either octanoic acid or decanoic acid (Figure 3.5). While the exogenously fed butyl ester substrates could be detected in both the culture supernatant and pelleted cell fractions (data not shown), the acid and alcohol breakdown products were detected primarily in the culture supernatant, with only trace amounts present in the pellet. This was not unexpected as it is known that both butanol and free fatty acids are diffused extracellularly by *E. coli* (Shen *et al.*, 2011; Lennen *et al.*, 2013). Therefore,

only the material in the culture supernatant was quantified. Figure 3.5 shows that the amount of butanol and decanoic acid in the supernatant of cultures supplemented with butyl decanoate was considerably lower than that of butanol and octanoic acid present in cultures supplemented with butyl octanoate. This difference is most likely the result of a combination of two factors, the first being the respective substrate specificity of the PFE enzyme for hydrolysing these two esters – with a preference for the shorter, octanoate chain length (Table 3.2) – and the second being the respective ability of these two esters to move across the plasma membrane. The proportion of butyl decanoate present in the culture supernatant was consistently higher than the proportion of butyl octanoate present in the supernatant at each time point sampled (data not shown), suggesting that the larger ester may move more slowly across the membrane into the cytoplasm. This would ultimately influence the intracellular concentration of butyl decanoate available to the PFE enzyme for hydrolysis. These results indicate that butyl-octanoate would be a more suitable end-product ester for butanol sequestration as it should move more easily across the plasma membrane – a desirable quality for this strategy.

Additionally, this confirmation that both butyl octanoate and -decanoate are capable of moving across the plasma membrane into the cytoplasm suggests that the lack of microbial toxicity observed for both of these esters even at high concentration in culture (Section 3.2.1) is a reflection of true molecular inertness, and not a result of their inability to interact with, and disrupt the function of, intracellular processes.

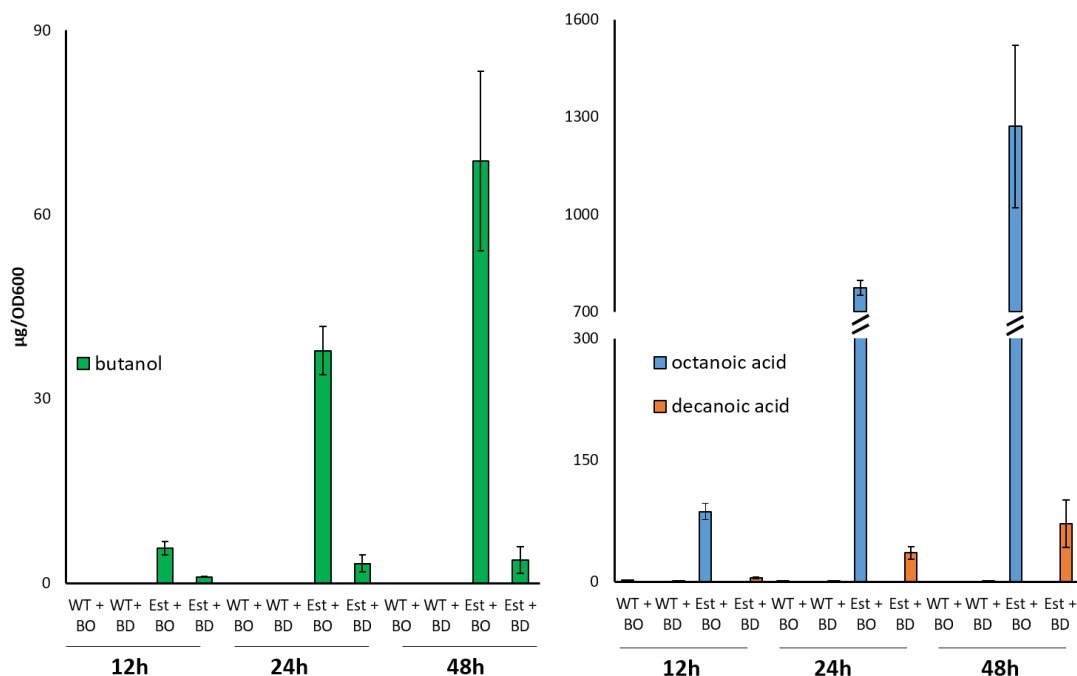


Figure 3. 5 Quantification of the butanol, octanoic acid and decanoic acid found in the supernatant of cultures of *E. coli* strain DH5 α heterologously expressing an esterase from *P. fluorescens* (PFE) at 12, 24, and 48 hours after feeding with either 10 mM butyl octanoate or -decanoate. Data are the mean + standard deviation from three biological replicates.

A potential criticism of the strategy employed above – with the heterologous expression of a non-secreted esterase in *E. coli* to confirm the movement of esters across the plasma membrane into the cytoplasm – is that it does not take into consideration the effects of cell lysis. While the PFE enzyme used in this work is non-secreted, it is possible that through cell lysis it becomes exposed to esters present in the extracellular milieu, hydrolysing them and ultimately confusing the analysis. Thus, depending on the amount of cell lysis taking place, this could skew the results to indicate these esters move across the plasma membrane more readily than they truly do. To account for hydrolysis due to enzyme released by cell lysis, two culture conditions were compared: (1) at 12 hours of growth post induction for PFE expression, either 10 mM butyl octanoate or -decanoate was fed into the culture and incubated for a further 12 hours before analysis, as done previously, and (2) at 12 hours of growth post induction for PFE expression, only the culture supernatant was isolated and incubated with either 10 mM butyl octanoate or decanoate for a further 12 hours before analysis of culture supernatant for breakdown products. Condition (2) allows for comparison of the amount of ester hydrolysis that occurs via

extracellular PFE activity as a result of cell lysis. Figure 3.6 shows that the amount of ester hydrolysis that can be attributed to extracellular PFE activity accounts for approximately 10% of the total butyl octanoate hydrolysis in culture and 30% of the total butyl decanoate hydrolysis in culture. Thus, the majority of breakdown product detected in the supernatant can be attributed to intracellular ester hydrolysis as opposed to extracellular hydrolysis, confirming that both these esters are able to move across the cell membranes into the cytoplasm.

Ultimately, there is very little known about the mechanism by which esters move across the plasma membrane into the cell, however these results would tentatively suggest that the size of the ester plays a role in the efficiency of the translocation – with longer chains moving across less proficiently. Though much further work would need to be done to confirm this, which is out of the scope of this project. For our purposes, this work has confirmed that both butyl octanoate and -decanoate are able to translocate across the plasma membrane, further supporting their use as end-product esters in our detoxification strategy.

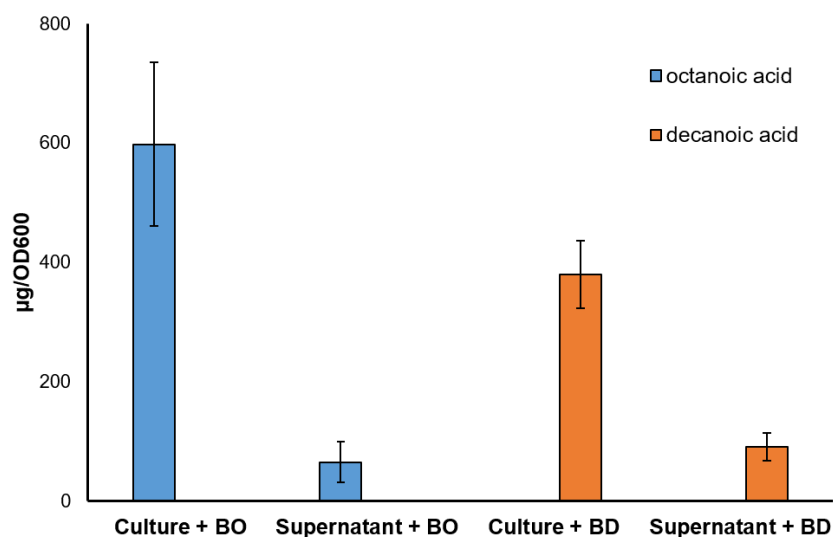


Figure 3. 6 Comparison of the amount of octanoic acid and decanoic acid found secreted into the extracellular milieu of either: cultures of *E. coli* strain DH5 α heterologously expressing PFE, or just the supernatant from *E. coli* strain DH5 α cultures expressing PFE. Breakdown products were quantified after 12 hours of sample incubation with either 10 mM butyl octanoate or -decanoate. Data are the mean \pm standard deviation from three biological replicates.

3.3 Conclusion

The preliminary work carried out here determined that the strategy of sequestering butanol into an ester molecule as a means to decrease microbial toxicity is a valid strategy. Toxicity tests aimed at identifying butyl esters that are more tolerated than butanol by *E. coli* identified two potential candidates, butyl octanoate and decanoate. In addition to causing no molecular or phase toxicity in culture, it was confirmed that both these butyl esters are able to move across the plasma membrane – an ideal feature with regards to downstream processing. Of these two butyl esters, the shorter chain octanoate ester was identified as a more suitable candidate for this strategy because, though it is equally as non-toxic as the decanoate ester, it appeared to move across the plasma membrane more easily. This is beneficial as it would mean (i) no further strain modification would be required to facilitate the export of our end-product ester, and (ii) no cost intensive fermentation extraction process would be required.

In this chapter, it was established that developing a *E. coli* platform that endogenously sequestration of butanol into an ester could offer a simple solution to the complex and multifaceted problem of microbial butanol toxicity. To put this strategy into practise, an enzyme capable of esterifying an alcohol and an acid – named an alcohol acyltransferase (AAT), will be required. Specifically, an AAT that is capable of esterifying butanol and octanoic acid to produce butyl octanoate.

4. Engineering *E. coli* for the production of medium chain butyl esters as a strategy for butanol sequestration

4.1 Introduction

Volatile short and medium chain esters (C4-C14) are found naturally in the flowers and ripening fruit of plants, where they play a dual function in contributing to the aroma and flavour of the plant as an attractant to animals, as well as acting as a deterrent towards microbes and pathogens (Beekwilder *et al.*, 2004; Rodriguez *et al.*, 2014). In addition, they can be produced during fermentation by certain species of yeast and lactic acid bacteria (LAB) – the results of which can be seen in beer and wine (Verstrepen *et al.*, 2003; Costello *et al.*, 2013). The enzyme responsible for the synthesis of these esters is called an alcohol acyltransferase (AAT), which catalyses the condensation of an alcohol with an acyl-CoA. These enzymes are capable of combining different acyl-CoA and alcohol substrates to produce a wide range of esters – which together contribute to the complexity of the aroma (Harada *et al.*, 1985; El-Sharkawy *et al.*, 2005). These enzymes are members of the BAHD acyltransferase family (an acronym for the first letter of the first four enzymes characterised in this family). Members of this family contain a conserved HXXXD catalytic motif and a DFGWG motif (Galaz *et al.*, 2013; Morales-Quintana *et al.*, 2013; Molina and Kosma, 2015). These enzymes typically have promiscuous activity for producing short chain esters, often with acyl chain lengths of C2 or C4. Table 4.1 shows all the characterised AAT enzymes to date which have been isolated and characterised for substrate specificity either by *in vitro* assay or heterologous expression. AAT activity has been identified in several other organisms, but the individual enzymes responsible have not been isolated.

A number of groups have utilized the heterologous expression of AAT enzymes for the production of these short and medium chain esters in *E. coli* and other model hosts with the intention of developing platforms for the microbial production of these compounds for cosmetics, perfumes, food/beverages, solvents, plasticizers, and biofuels (Vadali *et al.*, 2003; Park *et al.*, 2009; Rodriguez *et al.*, 2013; Guo *et al.*, 2014; Tai *et al.*, 2015). The choice of AAT enzyme employed in a given platform will depend on the host organism itself and the desired

end product ester(s). Thus far, the most commonly used AAT enzymes for these purposes have been ATF1 and ATF2 from *S. cerevisiae* and SAAT from *Fragaria x ananassa* because of their high activity and promiscuous substrate specificity (Table 4.1). In addition to the choice of AAT enzymes used, several additional considerations must be made when engineering a system for high titre production of a desired end-product ester. First, the alcohol and acid substrate availability in a chosen host. For example, *E. coli* is only able to natively produce a single alcohol, ethanol, and minimal amounts of short chain free fatty acids (Rodriguez *et al.*, 2013), and as such, this platform requires either exogenous substrate feeding (Horton *et al.*, 2005; Guo *et al.*, 2014), or further heterologous expression of the requisite pathways for endogenous substrate production. As exogenous culture feeding can often be limited by the microbial toxicity of a given compound, a number of groups have opted to engineer the endogenous production of desired short chain alcohol and acid substrates – such as butanol, isobutanol, isoamyl alcohol, and butyrate (Rodriguez *et al.*, 2013; Layton *et al.*, 2014; Tai *et al.*, 2015). Second, tailoring of the final ester profile being produced. AAT enzymes are often promiscuous and will synthesise a range of different esters in a heterologous host as opposed to a single end-product. Depending on the commercial application, either a single ester product may be desirable or a mixture containing certain proportions of constituent esters. As such, several groups have looked at modelling the structure of an AAT enzyme to identify strategies to broaden or narrow their substrate specificity (El-Sharkawy *et al.*, 2005; Lucchetta *et al.*, 2007). To date, there is no available crystal structure for an AAT enzyme. Third, often additional genetic manipulation of the host strain is required to minimize competing pathways for substrates and cofactors, and to engineer driving forces towards ester production (Valdali *et al.*, 2003; Rodriguez *et al.*, 2013; Layton *et al.*, 2014; Guo *et al.*, 2014). For example, Layton *et al.* (2014) utilized an *E. coli* strain with nine gene knock outs and one gene knock in that was designed to block major fermentative pathways, an inefficient electron transport system, fatty acid degradation, and the oxidative pentose phosphate pathway. With this strain, they were able to increase ethyl butyrate titres 14-fold compared to their base strain. Ultimately, through the use of heterologous AAT expression and further strain engineering, a number of microbial platforms for the production of short chain acetate and butyrate ester have been created.

In contrast, however, progress in the development of microbial platforms for the production of medium chain esters has lagged behind, with one of the limiting factors being the identification of AAT enzymes with substrate specificity towards medium chain acids and alcohols. While many AAT enzymes have been characterised with activity towards a diverse number of

substrates, their activity is typically highest for alcohol chain lengths of $\leq C4$, and acid chain lengths of $\leq C6$, and lower for larger and bulkier substrates (Table 4.1). Unfortunately, the medium chain ester butyl octanoate – our target end-product – falls within the latter category, and very few AAT enzymes have documented activity for synthesizing it. To date, three AAT enzymes from *Malus pumila* (Royal Gala apple), *Actinidia eriantha* (kiwi fruit) and *Actinidia chinensis* (kiwi fruit) have reported ability to synthesize butyl octanoate (Souleyer *et al.*, 2005; Günther *et al.*, 2011), and of these three, the AAT from *A. chinensis* has the highest reported activity. In this work, our strategy for detoxification of butanol via its esterification requires an AAT with high activity and substrate specificity, as this esterification step should not be a bottleneck reaction. Successful detoxification requires that butanol be efficiently sequestered into butyl octanoate to minimize alcohol accumulation in culture. As well, this strategy requires minimal promiscuity from the AAT enzyme for producing other carboxyl esters as it was determined previously that shorter chain butyl esters, such as butyl butanoate and -hexanoate, are inhibitory to *E. coli* growth at low concentrations (see chapter 3).

Here work is done towards engineering a strain of *E. coli* for the production of butyl octanoate as a strategy for the detoxification of endogenously produced butanol. To do this (i) an AAT enzyme from *A. chinensis* was expressed in *E. coli* that has activity for synthesizing butyl octanoate; (ii) optimize anaerobic butanol production in *E. coli* through heterologous expression of the butanol production pathway from *C. acetobutylicum*, (iii) investigate improving butyl octanoate production and minimizing side product formation in *E. coli* through engineering of both the AAT enzyme and *E. coli* metabolism; (iv) investigating bottlenecks in butyl octanoate synthesis in the butanol producing *E. coli* strain.

Table 4. 1 List of characterised AAT enzymes, their native host and their substrate specificity for alcohol and acyl-CoA substrates. Only enzymes that have been isolated, heterologously expressed, and characterised for esterification activity and substrate specificity have been included. The two letters in front of each AAT represent the host organism.

AAT enzyme	Organism	Alcohol chain length	Acid chain length	Reference
MpAAT1	<i>Malus pumila</i> (cv. Royal Gala)	-C3:0-OH, C4:0-OH, C6:0-OH, C10:0-OH	-C1:0, C2:0, C3:0, C4:0, C6:0,	Souleyer <i>et al.</i> , 2005

		-Hex-2-enol	and C8:0 CoAs	
		-(<i>E/Z</i>) hex-3-enol		
		-3 methylbut-3-enol		
		-2/3 methyl butanol		
		-Geraniol		
		-Furfuryl alcohol		
		-Benzyl alcohol		
		-2-phenyl ethanol		
AeAAT9	<i>Actinidia eriantha</i>	-C1:0-OH, C2:0-OH, C3:0-OH, C4:0-OH	-C2:0, C3:0, C4:0, C6:0, and C8:0 CoAs -Benzoyl CoA -2-(methylsulfyl) acetyl CoA	Günther <i>et al.</i> , 2011
AcAAT16	<i>Actinidia chinensis</i>	-C2:0-OH, C3:0-OH, C4:0-OH	-C2:0, C4:0, C6:0, and C8:0 CoAs -Benzoyl CoA -2-(methylsulfyl) acetyl CoA -2-(methylsulfyl) propanoyl CoA	Günther <i>et al.</i> , 2011
RhAAT	<i>Rosa hybrida</i>	-C2:0-OH, C4:0-OH, C6:0-OH, C8:0-OH Geraniol -Citronellol -Nerol -Linalool -Benzyl alcohol -2-phenylethyl alcohol	-C2:0 CoA	Shalit <i>et al.</i> , 2013

		-isoamyl alcohol		
		-cis-3-hexene-1-ol		
SAAT	<i>Fragaria x ananassa</i>	-C1:0 to C4:0-OH, and C5:0 to C10:0-OH	C2:0, C3:0, C4:0 and C6:0 CoAs	Aharoni <i>et al.</i> , 2000; Olias <i>et al.</i> , 2002; Beekwilder <i>et al.</i> , 2004
	var. Chandler	-2-propanol		
		-2-butanol		
		-Isoamyl alcohol		
		-(<i>E/Z</i>)-2-hexenol		
		-(<i>E/Z</i>)-3-hexenol		
		-Furfuryl alcohol		
		-Benzyl alcohol		
		-Cinnamyl alcohol		
		-2-phenyl ethanol		
		-Geraniol		
		-Nerol		
		-Eugenol		
		-Farnesol		
		-Perillyl alcohol		
VAAT	<i>Fragaria vesca</i>	-C1:0 to C4:0-OH, and C5:0 to C9:0-OH	-C2:0 CoA	Beekwilder <i>et al.</i> , 2004
		-Isoamyl alcohol		
		-(<i>E/Z</i>)-2-hexenol		
		-(<i>E/Z</i>)-3-hexenol		
		-Furfuryl alcohol		
		-Benzyl alcohol		
		-2-phenyl ethanol		
		-Geraniol		
		-Eugenol		

		-Perillyl alcohol		
		-2-ethoxyethanol		
		-Cinnamyl alcohol		
LAAT	<i>Citrus limon</i>	-C1:0-OH and C4:0-OH	-C2:0 and C3:0 CoA	Patent: WO2000032789 A1
FcAAT1	<i>Fragaria chiloensis</i>	-C4:0-OH	-C2:0 CoA	Gonzales <i>et al.</i> , 2009
SIAAT1	<i>Solanum lycopersicum</i>	-C3:0-OH to C6:0-OH -Isobutanol -(Z)-3-hexenol -2-methyl-1-butanol -3-methyl-1-butanol -Benzyl alcohol	-C2:0, C3:0, and C4:0 CoA	Goulet <i>et al.</i> , 2015
SpAAT1	<i>Solanum pennellii</i>	-C2:0-OH to C6:0-OH -Isobutanol -(Z)-3-hexenol -2-methyl-1-butanol -3-methyl-1-butanol -Benzyl alcohol 2-phenyl ethanol	-C2:0, C3:0, C4:0, and C6:0 CoA	Goulet <i>et al.</i> , 2015
CmAAT1	<i>Cucumis melo</i> var. <i>cantalupensis</i>	-C2:0-OH, C4:0-OH, C6:0-OH, C7:0-OH -Benzyl alcohol -Cinnamyl alcohol -2-phenylethanol -(E/Z)-2-hexenol -(E/Z)-3-hexenol -2-methyl propanol	-C2:0, C3:0, C4:0, and C6:0 CoAs	Yahyaoui <i>et al.</i> , 2002; Lucchetta <i>et al.</i> , 2007

		-2-methyl butanol		
		-3-methyl butanol		
CmAAT2	<i>Cucumis melo</i> var. cantalupensis	NA	NA	Lucchetta <i>et al.</i> , 2007
CmAAT3	<i>Cucumis melo</i> var. cantalupensis	-C4:0-OH -Benzyl alcohol -(Z)-2-hexenyl alcohol -3-methylbutanol -2-phenylethanol	-C2:0, C3:0, and C6:0 CoAs -2-methyl butanoyl CoA	Lucchetta <i>et al.</i> , 2007
CmAAT4	<i>Cucumis melo</i> var. cantalupensis	-C2:0-OH, C6:0 to C8:0-OH -(E/Z)-2-hexenyl alcohol -(E/Z)-3-hexenyl alcohol -Geraniol -Cinnamyl alcohol	-C2:0 and C4:0 CoAs	Lucchetta <i>et al.</i> , 2007
MaAAT	<i>Musa acuminata</i> var. Cavendish	-C3:0-OH, C5:0-OH, C6:0-OH -Isoamyl alcohol -3-hexenyl acetate	-C2:0 to C4:0 CoAs	Olias <i>et al.</i> , 1995
BanAAT	<i>Musa sapientum</i>	-C8:0-OH -(E)-2-hexenol - Geraniol -Nerol -Cinnamoyl alcohol	-C2:0 CoA	Beekwilder <i>et al.</i> , 2004
BEAT	<i>Clarkia breweri</i>	-Benzyl alcohol -Cinnamyl alcohol	-C2:0 CoA	Dudareva <i>et al.</i> , 1998

		-2-napthalene ethanol		
		-2-phenylethanol		
BEBT	<i>Clarkia breweri</i>	-C2:0-OH, C4:0-OH, C5:0-OH, C6:0-OH, C8:0-OH	-C2:0, C3:0, C4:0, C6:0, and C8:0 CoAs	D'auria <i>et al.</i> , 2002
		-2-phenylethanol	-Benzoyl-CoA	
		-Benzyl alcohol	-Cinnamoyl-CoA	
		-Cinnamyl alcohol		
		-Geraniol		
		-Linalool		
		-Cis-3-hexen-1-ol		
		-Anthranilate		
		-(p/m)-hydroxy benzyl alcohol		
OeAAT	<i>Olea europaea</i>	-C3:0-OH and C6:0-OH	-C2:0 CoA	Salas, 2004
		-3-methyl butanol		
		-(E/Z)-3-hexenyl alcohol		
BPBT	<i>Petunia hybrida</i>	-Isobutanol	-C2:0 CoA	Orlova <i>et al.</i> , 2006; Tai <i>et al.</i> , 2015
		-Isoamyl alcohol	-Benzoyl-CoA	
		-Benzyl alcohol		
VpAAT1	<i>Vasconcellea pubescens</i>	-C1:0-OH, C2:0-OH, C4:0-OH, C6:0-OH, C8:0-OH	-C2:0, C4:0, and C6:0 CoAs	Balbontín <i>et al.</i> , 2010
		-2-phenylethanol		
		-Benzyl alcohol		
		-Geraniol		
		-Cinnamyl alcohol		
AMAT	<i>Vitis labrusca</i>	-C1:0-OH, C2:0-OH, C4:0-OH, C6:0-OH,	-C2:0 CoA	Wang and De Luca, 2005

		C8:0-OH	-Benzoyl CoA	
		-2-propanol	-Anthraniloyl CoA	
		-Isobutanol		
		-Isopentanol		
		-Benzyl alcohol		
NsAAT	<i>Neurospora</i> sp.	-C1:0-OH to C6:0-OH, C3:1-OH, C5:1-OH	-C4:0 to C8:0, and C10:0 CoAs	Yamauchi <i>et al.</i> , 2014
			-Isovaleroyl-CoA	
ATF1	<i>S. cerevisiae</i>	-C2:0-OH, C3:0 to C8:0-OH	-C2:0, C4:0, C4:1, and C6:0 CoAs	Verstrepen <i>et al.</i> , 2003; Rodriguez <i>et al.</i> , 2014
		-Isobutanol		
		-2-methyl butanol		
		-3-methyl butanol		
		-2-phenylethanol		
ATF2	<i>S. cerevisiae</i>	-C3:0-OH, C5:0 to C8:0-OH	-C2:0 CoA	Verstrepen <i>et al.</i> , 2003
		-Isobutanol		
		-2-phenylethanol		
Lg-ATF1	<i>S. bayanus</i>	-C3:0-OH, C6:0-OH, C7:0-OH	-C2:0 CoA	Verstrepen <i>et al.</i> , 2003
		-Isobutanol		

4.2 Results

4.2.1 Production of butyl esters in *E. coli* by heterologous expression of an AAT from *A. chinensis*

The AAT gene sequence from *A. chinensis* was codon harmonized to the genome of *E. coli* (Materials and Methods 2.3.1) as a gene string, and subsequently cloned into the high copy pET21a plasmid between restriction sites *Bam*HI and *Xho*I to drive expression under the strong T7 promoter. From this point onwards, this harmonized AAT is referred to as *EcAAT* where *Ec* represents the gene's harmonization to the *E. coli* genome. Plasmid pET21a::*EcAAT* was transformed into *E. coli* strain C43 (DE3) and cultured as described by Souleyre *et al.*, (2005), with the exogenous addition of alcohol and acid substrate for butyl ester production. Two modifications were made to the original protocol (full protocol in materials and methods 2.5.6). The first modification was that TB+2% glucose media was used in the place of 2TY media. This was done as a comparison of the final butyl ester titres achieved from culturing in different medias showed that TB+2% glucose resulted in the highest titres (Appendix Supplementary Figure 3). The second modification made was that cultures were incubated for 18 hours instead of 20, this was done as a time course of butyl ester production over 21 hours showed that the highest titres were achieved at 18 hours (Appendix Supplementary Figure 4). Culturing conditions were optimized according to final butyl ester titres as opposed to *EcAAT* protein levels as it was not tagged for purification (it was found that the presence of a C-terminal His6x tag significantly reduced butyl ester formation by the AAT – data not shown), and expression was too low to distinguish from background proteins in either the soluble or insoluble extracts. For butyl octanoate production, cultures were exogenously fed 10 mM butanol and 5 mM octanoic acid 2 hours post induction, this was done to provide an excess of substrate for the AAT enzyme. Concentrations of octanoic acid above 5 mM were found to negatively impact culture growth rate (data not shown). Under these optimised expression conditions, *E. coli* was able to produce a mixture of short and medium chain butyl esters that included butyl butyrate, -hexanoate, and -octanoate (Figure 4.1). These esters were found in both the culture supernatant and cell pellet fractions that were obtained after centrifugation, with the proportion of ester

found in the pellet increasing as the chain length of the ester increased, i.e. approximately 11% of total butyl butyrate was found in the cell pellet, whereas 78% of total butyl octanoate was found in the pellet. This is possibly a result of the increased hydrophobicity of the longer octanoate ester, which allows it to partition more efficiently into the cell membrane compared to the more hydrophilic butyrate ester, but could also reflect the formation of a second phase intracellularly.

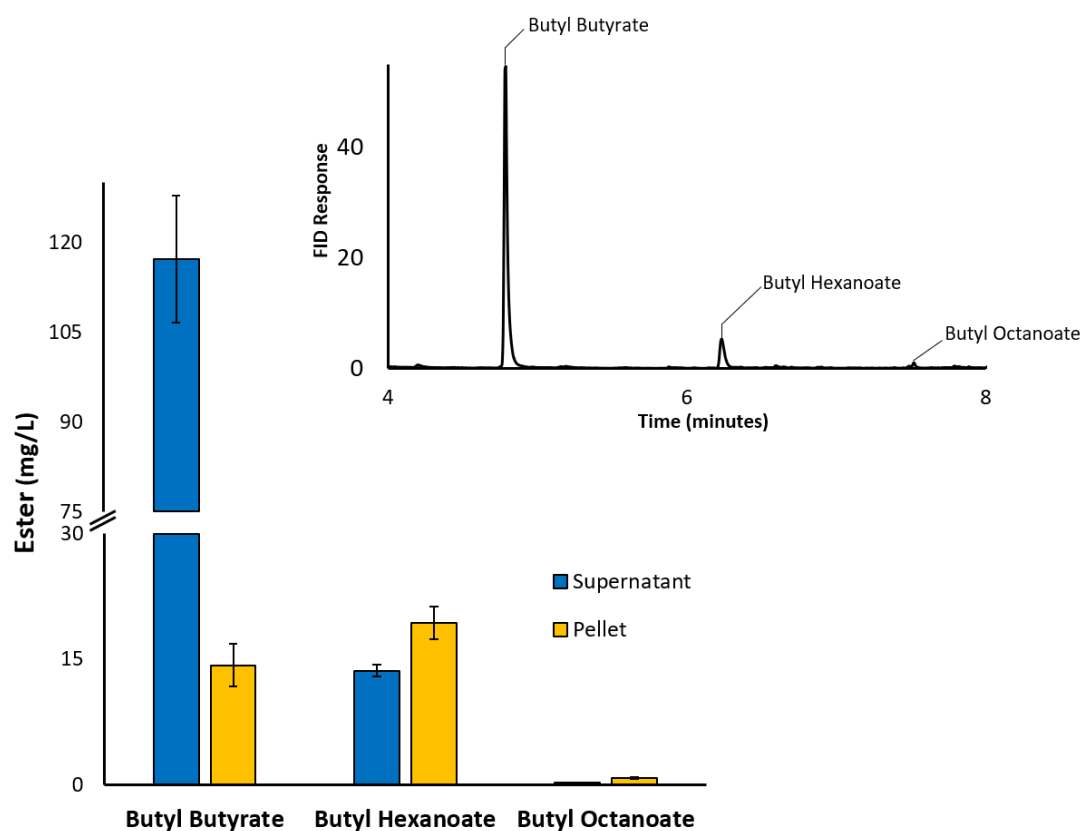


Figure 4. 1 The butyl ester profile found in the culture supernatant and cell pellet after centrifugation of *E. coli* expressing a codon harmonized AAT from *A. chinensis*. Cultures were induced with 0.4 mM IPTG and then fed 10 mM butanol and 5 mM octanoic acid before being further incubated at 20°C for 18 hours. Final average OD₆₀₀ was 31.6. Inset shows a gas chromatography trace of the butyl esters present in supernatant fraction. Butyl butyrate, hexanoate, and octanoate are indicated. Data are the mean \pm standard deviation of four replicates.

The presence of butyl butyrate and butyl hexanoate in *EcAAT* expressing *E. coli* cultures was not completely unexpected as this AAT enzyme has previously been shown to have activity for synthesising esters of these chain lengths (Günther *et al.*, 2012). Indeed, it has shown higher catalytic activity for making these shorter chain esters over butyl octanoate. As neither butyric acid nor hexanoic acid were exogenously fed into the culture this suggests that *EcAAT* is utilizing endogenously produced C4:0 and C6:0 acyl-CoAs to make these esters. A probable source for these shorter acyl chains is the intracellular degradation of the octanoic acid fed into the culture. As an extracellular free fatty acid is transported across the internal cell membrane into the cytosol by the FadD enzyme it is coupled with the esterification of the free acid to an acyl-CoA. This acyl-CoA is then a direct substrate for β -oxidation, which is the iterative breakdown of a fatty acyl-CoA by two carbon units for every cycle (Figure 4.17) (Jaßen *et al.*, 2014; Yao *et al.*, 2015). Therefore, in this system, the octanoic acid being fed into the culture is first being converted to octanoyl-CoA which is then acting both as a direct substrate for the *EcAAT* enzyme as well as being sequestered into the β -oxidation pathway where it is progressively broken down to hexanoyl-CoA and butyryl-CoA – both of which are also substrates for our AAT enzyme. The proportion of butyl octanoate produced compared to the shorter chain butyl hexanoate and -butanoate esters suggest that our *EcAAT* enzyme is not acting on the octanoyl-CoA as fast as it is being transported into the cell, so that the intracellular substrate is preferentially being metabolised by the fatty acid degradation pathway. This suggests that either the activity, or substrate specificity of this enzyme for octanoyl-CoA, will need to be improved for the success of our detoxification strategy. Interestingly, exogenous feeding of either hexanoic or butyric acid instead of octanoic acid into cultures of *E. coli* expressing the *EcAAT* resulted in significantly less ester formation, though they are preferred substrates of the *EcAAT* enzyme compared to the octanoic acid chain length (Figure 4.2). For example, cultures fed butyric acid produced approximately 6% of the amount of butyl butyrate compared to those cultures fed octanoic acid (Figure 4.2). This is perhaps due to the uptake rate of fatty acids by *E. coli*, which has been shown to decrease as the acid chain length decreases, with long-chain fatty acids (C12-C18) being preferred over medium-chain acids (Klein *et al.*, 1971), or the substrate specificity of FadD (the enzyme responsible for converting a free fatty acid into a fatty acyl-CoA thioester), which preferentially acts on longer chain fatty acids (Ford and Way, 2015). This might suggest that modulation of the β -oxidation pathway may be required downstream to improve uptake of exogenously fed octanoic acid. As an aside it should be noted that the AAT enzyme from *A. chinensis* was found to have highest catalytic activity for

producing butyl benzoate, and as such we considered whether butyl benzoate would be a more suitable end product for our detoxification strategy. An added benefit to using benzoyl-CoA as a substrate is that it is not a direct substrate for β -oxidation, as the benzene ring must first be reduced and cleaved (Egland *et al.*, 1997), so it would not be sequestered away from *EcAAT*

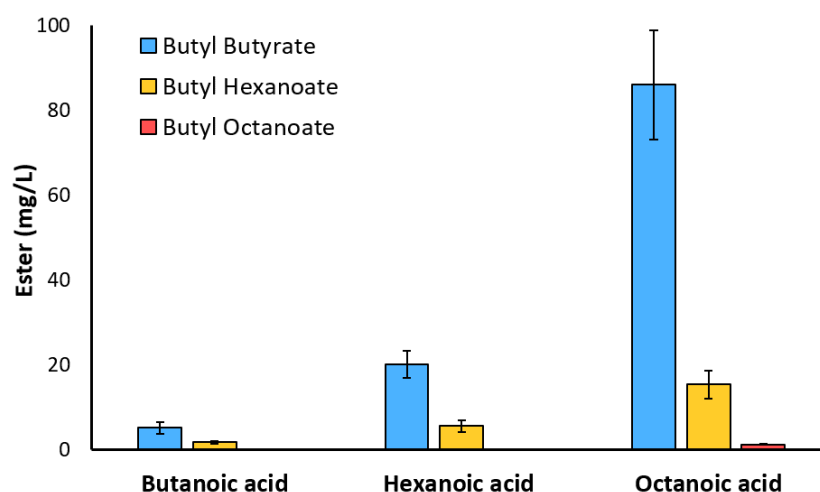


Figure 4. 2 The proportion of butyl butyrate, -hexanoate, and -octanoate present in the supernatant of *E. coli* cultures of strain C43 (DE3) expressing *EcAAT* when exogenously fed 10 mM butanol and 5 mM of either octanoic, hexanoic, or butyric acid. Supernatant was analysed 18 hours post induction. Data are the mean \pm standard deviation of three replicates

activity. However, it was found that butyl benzoate was not be an ideal end-product as toxicity tests revealed that it is inhibitory to *E. coli* growth at lower concentrations than butanol (appendix Supplementary Figure 2).

With confirmation that the *EcAAT* protein was capable of producing butyl esters in *E. coli* upon exogenous feeding of both the butanol and acid substrates, the next step was to integrate this enzyme into a system that endogenously produced butanol. Several groups have developed strains of *E. coli* capable of synthesising butanol through heterologous expression of the butanol production pathway from *C. acetobutylicum* (Atsumi *et al.*, 2008; Lee *et al.*, 2011; Shen *et al.*, 2011; Chin *et al.*, 2013; Wang *et al.*, 2015; Dong *et al.*, 2016). One such strain was gifted to the Leak lab by James C. Liao from the University of California (Los Angeles). This butanol

producing strain was a triple knockout of *E. coli* (JCL166) expressing two plasmids which together harbour the genes for the butanol production pathway (pEL11 and pIM8). While this strain will be described in detail in the following section (4.2.2), here the integration of the *EcAAT* gene in to this established butanol production platform is discussed. To do this, a three-plasmid system was developed in which plasmids one and two were pEL11 and pIM8 for butanol production, and the third plasmid was one that harboured the *EcAAT* enzyme. In constructing this three-plasmid system, two criteria required consideration; first, it was necessary that the third plasmid had an origin of replication (ori) that was compatible with those of pEL11 and pIM8 to avoid origin incompatibility. Origin incompatibility occurs when multiple plasmids within a single cell replicate by the same mechanism, and ultimately results in the loss of one of said plasmids (Velappan *et al.*, 2007). The origins of replication used by pEL11 and pIM8 are ColE1 and Cola, respectively, which are compatible with one another and both medium/high copy number. Thus, a third ori that is compatible with both those in pEL11 and pIM8, and which is also medium/high copy number, was required. Second, the third plasmid must possess a different antibiotic selection marker from those present in pEL11 and pIM8 so that it can be selected for, individually. pEL11 and pIM8 contain ampicillin and kanamycin resistance markers, respectively. Unfortunately, it was not possible to identify a previously constructed plasmid that satisfied these two requirements and thus, for this work, one was created. To do this, a pET21a plasmid was modified by replacing the original ColE1 ori with a p15A ori that is compatible with those present on pEL11 and pIM8 and results in a medium copy number, and the original ampicillin resistance marker was replaced with one for hygromycin.

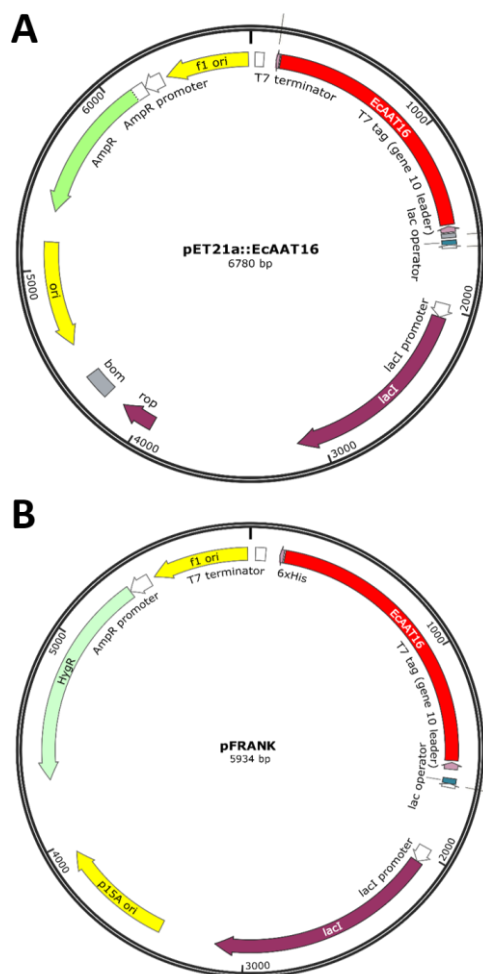


Figure 4. 3 Plasmid maps of **A** pET21a::EcAAT and **B** pFRANK. Plasmid pFRANK is a derivative of the pET21a vector where the antibiotic resistance marker was changed from ampicillin (light green) to hygromycin (light blue), and the origin of replication was changed from pColE1 to P15A (both in yellow). As well, the basis of mobility (bom) and rop protein sequence associated with the pColE1 origin of replication were removed in pFRANK.

A full description of how this was done with intermediate plasmid maps is described in Appendix Supplementary Figure 6. The resultant plasmid was named pFRANKENSTEIN (pFRANK for short) and fulfilled both criteria required for being compatible with the butanol pathway harbouring plasmids, as well as possessing the *EcAAT* gene (Figure 4.3). To confirm pFRANK was able to express the AAT enzyme and produce butyl esters, it was transformed into *E. coli* strain C43 (DE3), and cultured with butanol and octanoic acid supplementation 2 hours

post induction, as was done previously with pET21a::*EcAAT*. Figure 4.4 shows the resulting titres of butyl butyrate, -hexanoate, and -octanoate recovered from the supernatant of cultures expressing pFRANK. Here it can be seen that ester production was comparable to that achieved from pET21a::*EcAAT*, which produced 4.1 mg total ester/OD₆₀₀ after 18 hours, while pFRANK produced 3.3 mg total ester/OD₆₀₀. *E. coli* cultures harbouring pFRANK produced approximately 20% less total ester than those harbouring pET21a::*EcAAT*, with the assumption that this difference is attributed to the difference in plasmid copy number (with pET21a being high copy and pFRANK being medium copy) given the absence of any other noticeable difference between the two plasmids or expression conditions. Unfortunately, an ori that would be compatible with both plasmids pEL11 and pIM8 and also be high copy number could not be found.

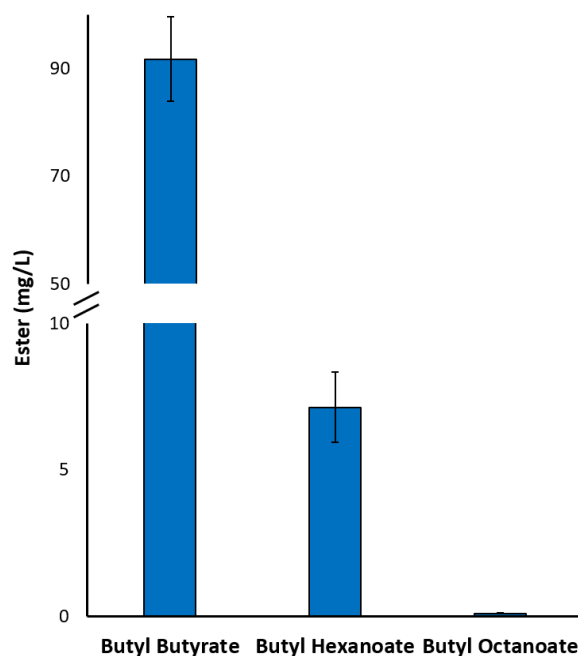


Figure 4. 4 Accumulation of butyl butyrate, - hexanoate and -octanoate in the supernatant of *E. coli* strain C43 (DE3) expressing pFRANK. Cultures were induced with 0.4 mM IPTG and grown for 18 hours at 20°C before analysis. Final average OD₆₀₀ was 30.1. Data are the mean + the standard deviation of three biological replicates.

4.2.2 Production of butanol in *E. coli* through heterologous expression of the *C. acetobutylicum* butanol pathway and the establishment of an NADH driving force

As mentioned previously in section 4.2.1, a butanol producing strain of *E. coli* constructed by the Liao lab at UCLA was acquired. There, they created a modified butanol production pathway that utilises an ‘artificial’ driving force to achieve high titre butanol production in *E. coli* (Shen *et al.*, 2011). The driving force that is taken advantage of is NADH, the primary reducing cofactor used throughout the butanol pathway. This driving force is established by deleting several mixed-acid fermentation reactions – those for the synthesis of lactate, ethanol, and succinate – that are NADH consuming reactions during anaerobic *E. coli* growth. The resulting strain, JCL166 ($\Delta ldh \Delta adhE \Delta frdBC$), is therefore no longer able to grow anaerobically due to its inability to recycle NADH (Figure 4.5). This cofactor build-up, therefore, creates a driving force for pathways that are NADH consuming. By expressing the NADH consuming butanol production pathway from *C. acetobutylicum* in this JCL166 strain, anaerobic growth is rescued and a driving force towards butanol production is established. To further couple the NADH driving force to the butanol pathway, the *bcd-efAB* gene responsible for the reduction of crotonyl-CoA to butyryl-CoA in the *C. acetobutylicum* butanol pathway was replaced with a *trans*-enoyl-CoA reductase (*Ter*) gene from *Treponema denticola*, who’s encoded protein carries out the same reduction reaction, but unlike *bcd-efAB*, does so in an irreversible fashion, and uses NADH directly as a reducing agent (*bcd-efAB* additionally requires ferredoxin). As all the other reactions steps in the butanol pathway are reversible, the introduction of the physiologically irreversible *Ter* catalysed step further strengthens the driving force to channel carbon flux towards butanol synthesis (Figure 4.5; Shen *et al.*, 2011). The JCL166 knockout strain, as well as plasmids pEL11 and pIM8 – which together harbour the butanol pathway genes – were provided by the Liao group. Plasmid pEL11 contains the *atoB*, *adhE2*, *crt*, and *hbd* butanol pathway genes from *E. coli* and *C. acetobutylicum*, and plasmid pIM8 contains the *Ter* gene from *T. denticola* (Figure 4.5).

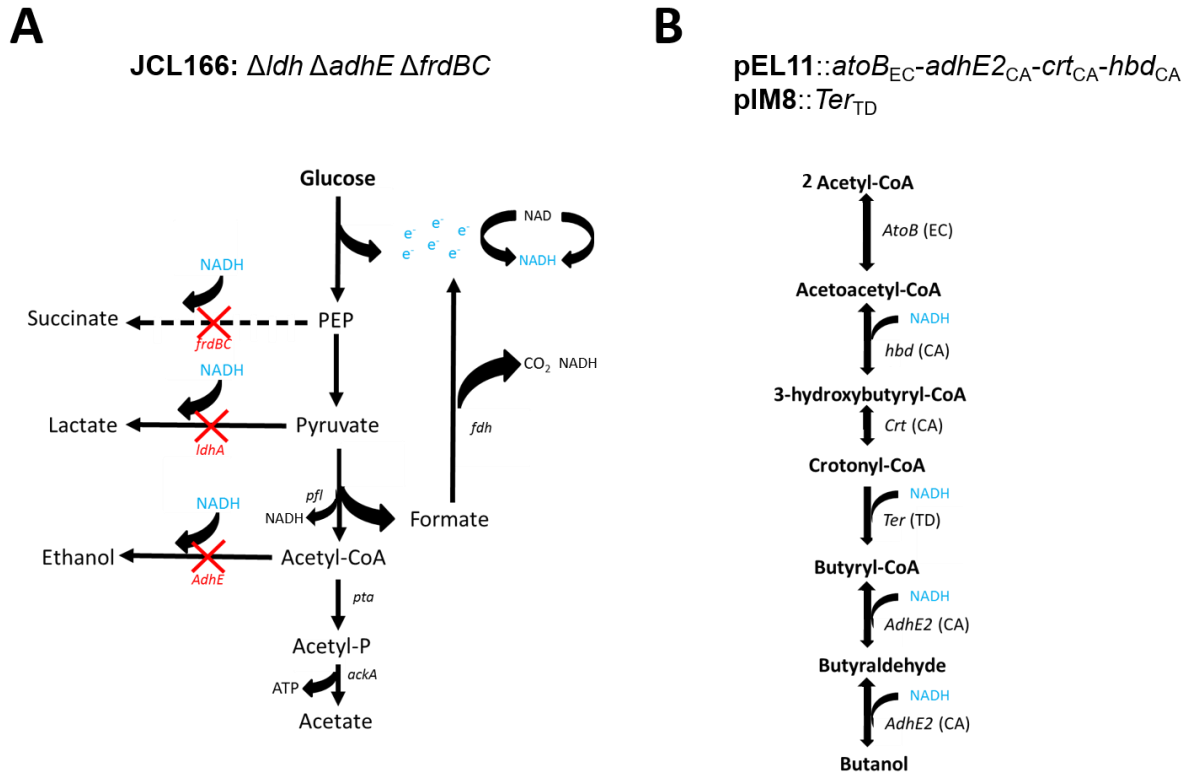


Figure 4. 5 Diagrams showing the engineered driving force leading towards improved butanol production in *E. coli*. **A** shows the modifications made to *E. coli* strain JCL166, which has three knockouts (*frdBC*, *ldhA*, and *adhE*). Each of these enzymes controls an NADH (shown in blue) consuming reaction, resulting in an NADH build up in the triple knockout strain. **B** the 1-butanol production pathway expressed in *E. coli* from *C. acetobutylicum*. This pathway utilises NADH as an electron donor, providing a sink for the build-up of this cofactor in strain JCL166, leading to driving force towards butanol production. The organism from which heterologously expressed genes have been sourced are in brackets next to the gene name: CA, *C. acetobutylicum*; EC, *E. coli*; and TD, *Treponema denticola*. Modified from Shen *et al.*, 2011.

A time course of butanol production from *E. coli* strain JCL166 transformed with pEL11 and pIM8, named strain DLB, was performed. Cultures were grown aerobically in glass vials to an OD of 0.8, induced with IPTG and grown an additional 2 hours aerobically to increase cell density before being flushed with nitrogen gas (N_2) and crimp-capped to maintain a micro-aerobic/anaerobic environment. The JCL166 knockout strain demonstrated retarded growth rate compared to wild type *E. coli*, and – even with the additional two hour incubation time between induction and the nitrogen flush – did not exceed an OD_{600} of 2. Butanol production and culture

OD₆₀₀ were analysed at intervals of 24 hours for a total of 96 hours for strain DLB (Figure 4.6). Over this time course, butanol production by the DLB strain continued to increase to a maximum of 245 mg/L at 96 hours, however, the culture OD₆₀₀ dropped, perhaps as a result of a drop in culture pH as the mixed-acid fermentation reactions that have not been knocked out in this strain produce acetate and formate. Though not measured here, Shen *et al.*, (2011), showed that culture pH dropped from 7 to approximately 5.4 over a similar 96 hour time course using this butanol producing strain. Concerningly, however, while butanol was successfully produced by strain DLB, titres were much lower than reported in the literature. At 24 hours Shen *et al.*, (2011) were able to produce approximately 500 mg/L butanol under microaerobic conditions, whereas here only 125 mg/L of butanol was achieved, a four-fold reduction in titre (Figure 4.6). 125 mg/L butanol is approximately 1.7 mmol/L, well below the toxicity limit and significantly less than the 10 mmol/L of butanol previously being fed into cultures expressing the *EcAAT*. In those exogenously fed cultures, analysis after 18 hours showed only trace amounts of butanol, suggesting that any lesser amount would be limiting to ester production. A number of optimization attempts were carried out to improve butanol titre, these involved varying the time of induction, IPTG concentration, length of aerobic incubation before N₂ flush, etc. However, no improvement on titre was made. As a result of this, it was hypothesized that a condition that could not be controlled using the crimp cap culture technique – such as pH, glucose availability, anaerobicity – was responsible for limiting butanol production. Therefore, a small bioreactor fermentation of strain DLB was run where all conditions would be monitored and maintained for optimal butanol production. Unexpectedly, butanol titres were not improved (data not shown), suggesting that the limiting factor was to do with the pathway itself, rather than the culturing conditions. It was previously shown that in a strain of JCL166 harbouring only the pEL11 plasmid, some butanol was produced (Shen *et al.*, 2011). Therefore, it was possible that the low level of butanol production could reflect poor expression of the *Ter* gene present on pIM8. Sequencing of the *Ter* gene showed no errors in the nucleotide sequence suggesting that, if expression was a problem, the fault may lay in the pIM8 backbone.

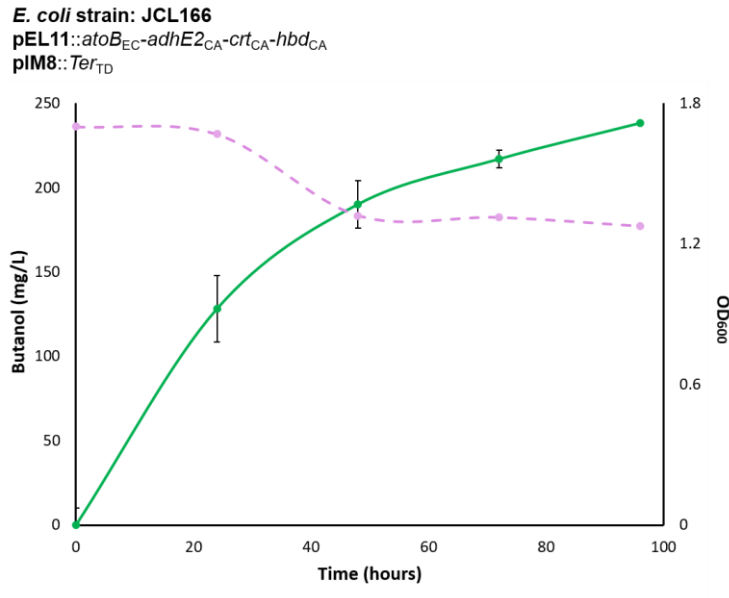


Figure 4. 6 Anaerobic fermentation of *E. coli* strain JCL166 harbouring plasmids pEL11 and pIM8 over the course of 96 hours. Cultures were flushed with nitrogen (N₂) and crimp capped three hours after induction with IPTG and grown at 30°C. The green line represents the amount of butanol present at each 24 hour time point, and the hashed mauve line represents the culture OD₆₀₀ over the course of the fermentation. Data are the mean \pm standard deviation of four biological replicates.

To confirm this hypothesis, the *Ter* gene, as well as a formate dehydrogenase (*Fdh*) gene from *Candida boidinii*, were synthesised – with RBS (ribosomal binding site) sequences directly upstream of each gene – and together inserted by Gibson assembly into pFRANK (a plasmid we had previously confirmed was able to express inserted genes) directly downstream of the *EcAAT* gene. Gibson assembly was used for this insertion as the only available restriction site downstream of the *EcAAT* gene was *Xho*I, and unlike digestion/ligation cloning, Gibson assembly would ensure that the *Ter* and *Fdh* genes would be inserted in the correct orientation. This new plasmid was named pVICTOR (Figure 4.7), and in addition to the *Ter* and *Fdh* genes, several additional restriction site were inserted downstream of the *Fdh* gene to allow for flexibility with any future gene insertion (Figure 4.7). *Fdh* is responsible for the oxidation of formate to CO₂ and NADH, and its over expression further increases the intracellular NADH driving force (Figure 4.5; Berrios-Rivera *et al.*, 2002; Nielsen *et al.*, 2009; Shen *et al.*, 2011). It has been shown previously by Shen *et al.*, (2011) that the introduction of the *Fdh* protein from

C. boidinii into the JCL166 system resulted in a slight increase in butanol production. To determine whether Ter activity was recovered when expressed on plasmid pVICTOR, it was transformed into *E. coli* JCL166 with pEL11 (pIM8 was excluded), and this new strain was named DLBO1. Cultures were grown as described previously for strain DLB in crimp capped glass vials, and butanol production was analysed after 24 hours of anaerobic growth. Butanol production was greatly improved in this new system where the *Ter* gene was expressed from pVICTOR instead of pIM8. These cultures produced butanol titres of approximately 400 mg/L at 24 hours (Figure 4.7). While this was slightly less than the literature value after 24 hours of culturing, it was still a significant improvement over the 125 mg/L achieved with the pEL11 and pIM8 system. These results confirm that expression of *Ter* from pIM8 was a problem, and that by moving it to pVICTOR, good expression was restored. This is additionally beneficial as by eliminating the need for the pIM8 plasmid the butyl ester production platform was now a two-plasmid system, instead of the three-plasmid system that was originally envisaged. This ultimately relieves some of the metabolic burden associated with maintaining and copying multiple vectors (Karim *et al.*, 2013).

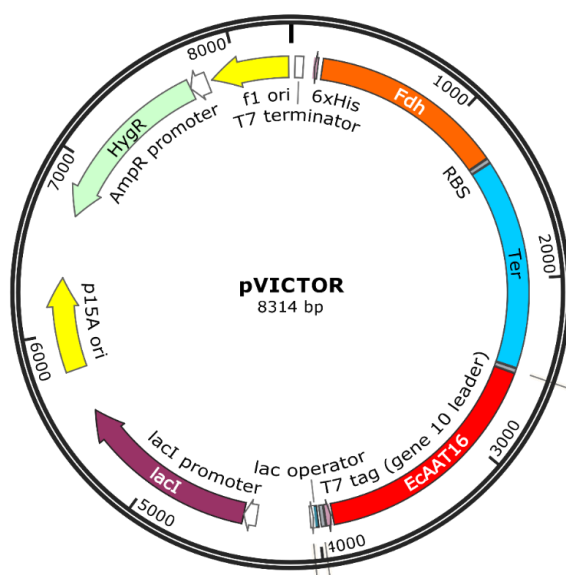


Figure 4. 7 Plasmid map of pVICTOR, a derivative of pFRANK. A *Ter* gene from *T. denticola* and an *Fdh* gene from *Candida boidinii* were cloned in downstream of the *ECAAT* gene. *Ter* and *Fdh* were inserted using Gibson assembly and possess a *KpnI* restriction site between them. Downstream of *Fdh* several addition restriction cut sites were added: *SalI*, *SacI*, and *NotI*.

In JCL166
pEL11::*atoB*_{EC}-*adhE2*_{CA}-*crt*_{CA}-*hbd*_{CA}
pVICTOR::*EcAAT*_{AC}-*ter*_{TD}-*fdh*_{CB}

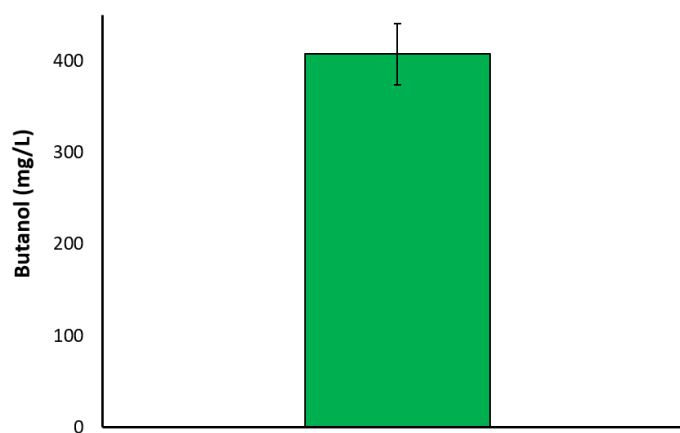


Figure 4. 8 Anaerobic butanol production from *E. coli* strain JCL166 harbouring plasmids pEL11 and pVICTOR. Cultures were induced with 0.1 mM of IPTG and grown aerobically for an additional 2 hours before being flushed with N₂ and crimp capped. Cultures were then analysed after 24 hours of anaerobic growth. Data are the men \pm standard deviation of four biological replicates.

Ultimately, the *E. coli* butanol production system developed by the Liao lab was successfully recreated. While the butanol titres achieved here were slightly lower than the literature values, this most likely reflected different culturing condition and perhaps an in-house difficulty in maintain cultures as micro aerobic/anaerobic when grown in vials. From this point, it was possible to move forward to the next step of investigating this detoxification strategy, which was the expression of the *EcAAT* in this butanol production background.

4.2.3 Butyl ester production in an *E. coli* strain harbouring the butanol synthesis pathway

The next step of this detoxification strategy was to initiate butyl octanoate production via the *EcAAT* enzyme in the context of the butanol producing *E. coli* strain to assess the success of

butanol sequestration into an ester molecule for detoxification. In this strategy, the source of the octanoate substrate could either be from culture medium supplementation with octanoic acid, genetic modifications to increase the intracellular availability of octanoyl-CoA, or a combination of both techniques. Economically, following downstream recovery and hydrolysis of the butyl octanoate for the production of butanol, the octanoic acid by-product should be recycled as a substrate for future expression cultures. As a preliminary experiment, prior to the creation of pVICTOR with improved Ter expression, *E. coli* strain JCL166 was transformed with the initial three-plasmid system of pEL11, pIM8 and pFRANK. This strain was named DLBO2. Strain DLBO2 was cultured aerobically as described above in section 4.2.1, including media supplementation with both butanol and octanoic acid. Butanol supplementation was necessary during aerobic culturing of strain DLBO2 as butanol production via the synthetic butanol pathway is driven by the need to re-oxidize NADH that arises during oxygen limited conditions when mixed-acid fermentation is typically triggered (Wen and Shen, 2016). Analysis of the supernatant of strain DLBO2 at 18 hours showed the presence of all three butyl esters (Figure 4.9), however the final titre of each ester was notably lower than the final titres produced by cultures of *E. coli* C43 (DE3) expressing either pET21a::*EcAAT* or pFRANK alone. From the supernatant of strain DLBO2, approximately 7 mg/L butyl butyrate, 0.8 mg/L butyl hexanoate, and 0.2 mg/L butyl octanoate was recovered (Figure 4.9); this is nearly 10-fold less than the titre seen with *E. coli* strain C43 (DE3) expressing pFRANK (Figure 4.9). As well, it was noted that at the point of analysis that the proportion of residual butanol present in the supernatant was significantly higher than previously observed in cultures of *E. coli* C43 (DE3) expressing either pET21a::*EcAAT* or pFRANK – in which it was nearly completely consumed. While cultures of DLBO2 did not quite reach the same cell density and those of C43 (DE3) expressing either pET21a::*EcAAT* or pFRANK – with an average OD₆₀₀ of 26.9 – the difference in culture density was not enough to explain the observed difference in ester production. Cultures of *E. coli* strain C43 (DE3) expressing pFRANK produced 3.3 mg total ester/OD₆₀₀ after 18 hours, while cultures of strain DLBO2 produced only 0.3 mg total ester/OD₆₀₀ after 18 hours. The low butyl ester titres achieved by strain DLBO2, paired with the high proportion of residual butanol at the point of analysis at 18 hours, suggested that *EcAAT* activity in this strain is not sufficient to incorporate butanol into an ester as quickly as those strains of C43 (DE3) expressing either pET21a::*EcAAT* or pFRANK alone. Further, strain DLBO2 cultured anaerobically, as described previously for strains DLB in section 4.2.3, with the addition of only 5 mM octanoic acid immediately before the anaerobic switch (no exogenous butanol was fed into cultures grown

anaerobically) was analysed 18 hours after the N₂ flush, and showed low levels of butanol and 0.6 mg/L butyl butyrate in the supernatant. No other butyl esters could be detected in either the supernatant or cell pellet (data not shown).

It may be that the reduced activity of *EcAAT* when expressed in strain DLBO2 compared to its activity when expressed in strains of *E. coli* C43 (DE3) harbouring either pET21a::*EcAAT* or pFRANK, alone, is a result of a metabolically stressed system. DLBO2 is composed of *E. coli* strain JCL166 – which possesses three gene knock-outs and a single gene knock-in strain – maintaining three plasmids (pEL11, pIM8 and pFRANK) which together hold the six genes required for butyl ester production, in addition to expressing resistance to four antibiotics. All together this places a large metabolic load on the host that can result in lower heterologous protein expression (Glick, 1995). Protein expression of *EcAAT* was already shown to be low in *E. coli* strain C43 (DE3) expressing pET21a::*EcAAT*, as a band for the *EcAAT* protein could not be resolved on SDS-PAGE gels (Appendix Supplementary Figure 5). While this was the original hypothesis to explain the decreased butyl ester formation from *E. coli* strain DLBO2, experiments that followed these experiments contradicted this explanation.

Following this preliminary work, and the creation of pVICTOR for improved Ter expression, it was necessary to confirm that expression of pVICTOR continued to produce butyl ester titres that were comparable to its parent plasmid, pFRANK – as had been done previously with pFRANK and pET21a::*EcAAT*. To do this *E. coli* C43 (DE3) was transformed with pVICTOR and cultured aerobically as described in section 4.2.1, including exogenous substrate addition. Unexpectedly, this strain produced only trace amounts of butyl butyrate in the supernatant and butyl octanoate and the cell pellet (Figure 4.10), with no improvement in titre regardless of attempts of optimization. Nucleotide sequencing of the *AAT* gene, and regions directly

In JCL166

pEL11::*atoB*_{EC}-*adhE2*_{CA}-*crt*_{CA}-*hbd*_{CA}

pIM8::*Ter*_{TD}

pFRANK::*EcAAT*_{AC}

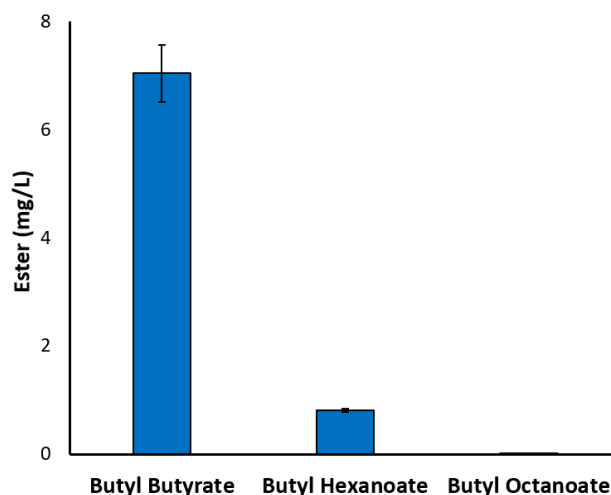


Figure 4. 9 Quantification of butyl esters present in the supernatant of cultures of *E. coli* strain DLBO2 (JCL166 harbouring plasmids pEL11, pIM8, and pFRANK) grown aerobically. Cultures were induced at an OD₆₀₀ of 0.8 and incubated for 2 hours before being exogenously fed 10 mM butanol and 5 mM octanoic acid. Cultures were grown for 18 hours at 20°C before analysis. Final average OD₆₀₀ was 26.9. Data is the mean \pm standard deviation of three biological replicates.

upstream and downstream of the *AAT* gene, showed no changes to the nucleotide sequence. Furthermore, it had previously been shown (section 4.2.2) that the *Ter* gene downstream of the *EcAAT* in pVICTOR was being successfully expressed, suggesting that the plasmid was functional. In literature reports, heterologous expression of AAT enzymes has been notoriously temperamental, with some AAT enzymes forming protein aggregates when expressed in *E. coli* (though enzyme activity was not completely lost, but rather reduced most likely due to occlusion of active sites), and some only expressing in certain strains of *E. coli* (Souleyre *et al.*, 2005; Zhu *et al.*, 2015). With this *EcAAT*, it was found that activity in *E. coli* was increased with the removal of a C-terminal His6x tag (data not shown). Considering that the only difference between the pVICTOR plasmid and its parent plasmid, pFRANK, was the addition of the *Ter* and *Fdh* genes downstream of the *EcAAT* within the same operon, it appeared that the transcription of *EcAAT* as part of an operon was compromising its translation, possibly due to

the formation of secondary structure. Thus, in an effort to bypass this problem, the *AAT* gene was removed from the operon by inserting a transcriptional terminator directly downstream of the *AAT*, and a second T7 promotor in front of the *Ter* and *Fdh* genes. This plasmid was named pVICTOR 2.0 and contained two operons, one of which expresses just the *EcAAT*, and a second that expresses the *Ter* and *Fdh* genes (Figure 4.11). With *EcAAT* removed from the operon, it was hoped that activity would be restored to that of the parent plasmid, pFRANK. Unfortunately, AAT expression from this plasmid resulted in no improvement in butyl ester production over pVICTOR (data not shown), and *E. coli* C43 (DE3) cultures transformed with pVICTOR 2.0 continued to produce only trace amounts of ester *in vivo*. As well, *in vitro* assays of AAT activity from the crude cell extracts of cultures expressing either pFRANK or pVICTOR 2.0 showed an approximately 55-fold reduction in product formation from AAT expressed from the latter plasmid (Table 4.2). In contrast, *in vitro* *Ter* activity from cultures expressing pVICTOR 2.0 was comparable to literature values (Table 4.2; Shen et al., 2011). Therefore, the hypothesis that *EcAAT*'s low activity was a result of its incorporation in an operon was incorrect, as it continued to have low activity when removed from the operon.

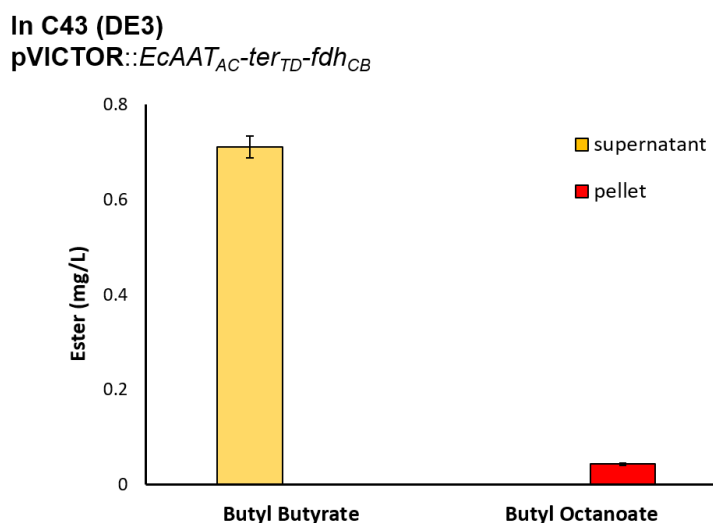


Figure 4. 10 Quantification of butyl esters present in the supernatant and pellet of *E. coli* C43 (DE3) transformed with plasmid pVICTOR. Cultures were induced at an OD₆₀₀ of 0.8 and incubated for 2 hours before being exogenously fed 10 mM butanol and 5 mM octanoic acid. Cultures were grown for 18 hours at 20°C before analysis. Data is the mean \pm standard deviation of three biological replicates.

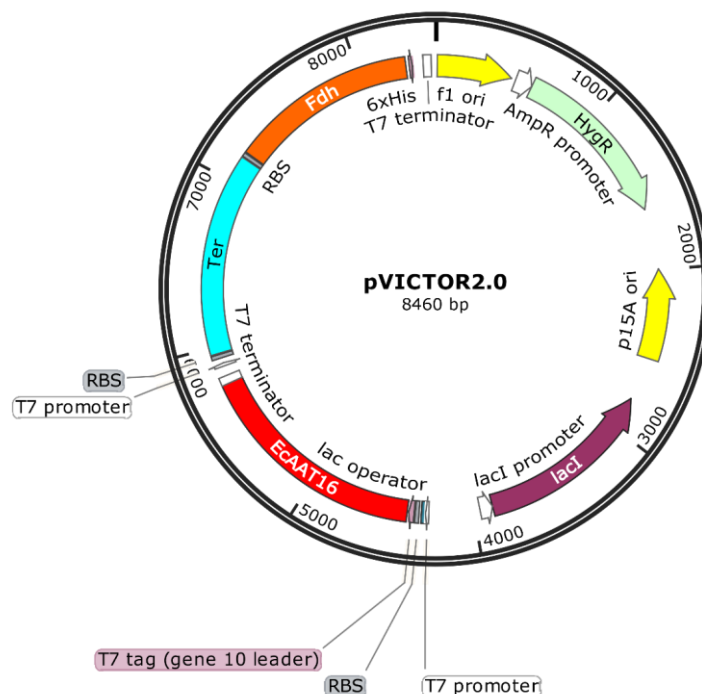


Figure 4. 11 Plasmid map of pVICTOR2.0, a derivative of pVICTOR. A terminator was inserted behind the *EcAAT* gene to remove it from the operon. A second T7 promoter and was inserted upstream of the *Ter* gene to initiate separate transcription of the *Ter* and *Fdh* genes.

Thus, it appeared that the activity of *EcAAT* was significantly reduced when expressed in combination with the *Ter* and/or *Fdh* genes, suggesting that one or both was in some way responsible for the reduced alcohol acyltransferase activity, either at the mRNA or protein level. However, work done by the Trinh group at the University of Tennessee suggested that it was most likely the *Ter* that was hindering alcohol acyltransferase activity, rather than the *fdh*. In their work, they expressed the SAAT alcohol acyltransferase from *F. ananassa* in *E. coli* to make ethyl butyrate in two separate systems. In one system, they expressed the SAAT along with the requisite genes to produce ethanol and exogenously fed in butyric acid. There they produced approximately 140 mg/L of ethyl butyrate (Layton *et al.*, 2016). In the second system, they expressed the SAAT along with the requisite genes to produce butyrate, including the *Ter* from *T. denticola*, and exogenously fed in ethanol. There they made just 6 mg/L of ethyl butyrate (Layton *et al.*, 2014). In both this work and ours, co-expression of the *Ter* gene and an AAT gene appears to drastically reduce ester production by the AAT protein, but neither butanol

(our work) or butyrate (Layton *et al.*, 2014) production via the Ter enzyme. Ter inhibition of AAT activity may also explain the low butyl ester production observed for strain DLBO2 cultured aerobically (Figure 4.9). This strain possessed the *Ter* gene on plasmid pIM8, a plasmid which was previously suspected to have either low or no expression of the Ter gene. It may be the case that, in fact, that there is a small amount of *Ter* expression from pIM8, which would account for the above basal levels of butanol production by strain DLB compared to a strain of JCL166 harbouring only the pEL11 plasmid (Figure 4.6, Shen *et al.*, 2011), as well as the significant reduction in butyl ester production of strain DLBO2. Ultimately, this evidence suggests that expression of the *Ter* gene in some way inhibits AAT activity, however, the means by which this occurs was unclear.

Table 4. 2 *In vitro* activity of Ter and AAT in crude cell extracts of wild type *E. coli* C43 (DE3), and *E. coli* C43 (DE3) harbouring either pFRANK or pVICTOR 2.0. Activity is the mean \pm standard deviation of four replicates.

	Activity ($\mu\text{mol product/min/mg crude protein}$)	
	Ter ^a	AAT ^b
C43 (DE3)	0.22 \pm 0.003	--
pFRANK	0.16 \pm 0.01	9.34x10 ⁻² \pm 8.8x10 ⁻³
pVICTOR 2.0	2.31 \pm 0.14	1.65x10 ⁻³ \pm 1.9*10 ⁻⁴

^a Ter assay performed at 30°C and 340 nm using 200 μmol NADH, 2 μmol FAD, and 200 μmol crotonyl-CoA

^b AAT assay performed at 30°C for 30 minutes using 10 mM butanol and 0.75 mM hexanoyl-CoA

With the evidence that Ter expression was in some way hindering AAT activity, it was necessary to identify at what point this inhibition was occurring; whether it be at the transcriptional level, translational level, or post-translational interference with enzyme activity. The possibility of transcriptional interference of the *EcAAT* gene was initially examined using RT-qPCR to compare *EcAAT* transcript abundance when expressed from plasmid pFRANK

versus pVICTOR 2.0 (Figure 4.12 A), as well as the transcript abundance of the *EcAAT* and *Ter* when both expressed from plasmid pVICTOR 2.0 (Figure 4.12 B). Results represent the fold change in target gene expression compared to a wildtype *E. coli* C43 (DE3) harbouring no plasmid (no template) control – which did have a low fluorescence signal, but not more than a no cDNA control. This low fluorescence signal may have been due to primer dimer formation or non-specific annealing; however, no other products were discerned through melt curve analysis or end-product analysis by DNA agarose gel (data not shown). This wildtype *E. coli* C43 (DE3) control, to which fold change of target gene expression was normalized to, was done to ascertain whether observed expression from each plasmid was valid, or no different from background. Transcript abundance of the *EcAAT* or *Ter* under all conditions have been normalised to the expression levels of the reference gene, 16S ribosomal RNA (*rrsA*) (Zhou *et al.*, 2011; Peng *et al.*, 2014). From panel A, it can be observed that *EcAAT* transcript abundance in *E. coli* C43 (DE3) expressing pFRANK is nearly 4.8-fold higher than the no template control, whereas *EcAAT* transcript abundance in *E. coli* expressing pVICTOR 2.0 is only 1.4-fold higher, indicating it is being expressed only slightly above background levels. Considering that background expression represents the signal produced from no expression of AAT, the results suggest that *EcAAT* expression from plasmid pVICTOR 2.0 is minimal. As well, transcript abundance of *EcAAT* and *Ter* from cultures expressing pVICTOR 2.0 were compared (Figure 4.12 B) and it was found that *Ter* expression from this plasmid is 4.5-fold higher than the no template control, whereas *EcAAT* is only 1.6-fold higher, which is again only slightly above background levels. In all cases it would be expected that the level of gene expression would be the same for *EcAAT* and *Ter* regardless of the plasmid they are expressed from as both genes are under the control of a T7 promotor, however these results demonstrate that *EcAAT* gene expression is significantly reduced when co-expressed with the *Ter* gene. However, that the fold change in expression of the *Ter* gene from pVICTOR2.0 is comparable to *EcAAT* expression from pFRANK suggests that expression of *Ter* is unaffected by its co-expression with *EcAAT*. Ultimately, these results suggest that the *EcAAT* is being inhibited at the transcriptional level by the *Ter* gene a unidirectional manner, however the mechanism by which this is occurring is still unknown. Interestingly, this phenomenon may not be unique to our *EcAAT*, as a similar decrease in ester product formation was observed in *E. coli* cultures co-expressing *Ter* and the *SAAT* from *F. ananassa* (Layton *et al.*, 2014).

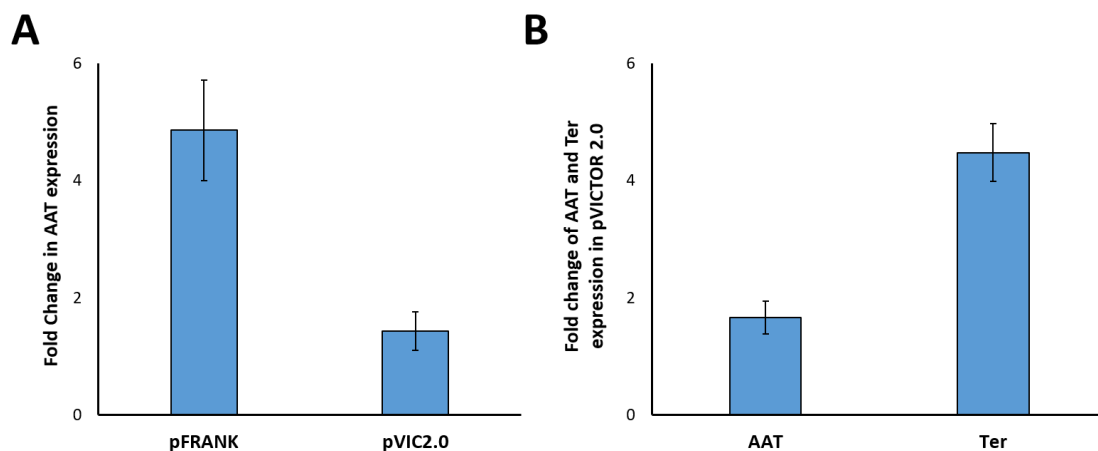


Figure 4.12 Fold change in expression of **A** the *EcAAT* gene in *E. coli* strain C43 (DE3) transformed with either plasmid pFRANK or pVICTOR 2.0, **B** fold change in expression of either the *EcAAT* or *Ter* gene in *E. coli* strain C43 (DE3) transformed with pVICTOR 2.0. Values are given as fold change in expression relative to wildtype signal – the no template control (=1.0) – using 16S ribosomal RNA (*rrsA*) as a reference gene. Data is the mean \pm standard deviation of three biological replicates.

4.2.4 Engineering substrate specificity for improved butyl octanoate production

An on-going and fundamental problem with using the *EcAAT* protein in this butanol detoxification strategy is the substrate specificity of this enzyme. Like most AATs, it has a broad substrate specificity (Table 4.1), and when expressed in *E. coli* supplemented with butanol and octanoate it produced several additional butyl esters in addition to the desired butyl octanoate end-product. Ultimately this is detrimental to this detoxification strategy, as the shorter chain length butyl esters (butyl butyrate and -hexanoate) are toxic to *E. coli* at low concentrations. Additionally, degradation of the octanoate will affect the economics of the proposed fatty acid recycling step. Therefore, methods for improving the product specificity of this system were considered, and two strategies were employed: (i) protein engineering of the *EcAAT* enzyme to increase its substrate preference for the octanoic acid chain length, and (ii) engineering of endogenous *E. coli* metabolism to increase the abundance of intracellular octanoyl-CoA.

4.2.4.1 Rational protein engineering

Alcohol acyltransferase proteins are members of the BAHD superfamily (named after the first four biochemically characterised enzymes in this family from plants) that is composed of acyltransferase enzymes involved in the synthesis of secondary metabolites such as anthocyanines, esters, phytoalexins, etc. (El-Sharkawy *et al.*, 2005; Lucchetta *et al.*, 2007; Galaz *et al.*, 2013; Navarro-Retamal *et al.*, 2016). Members of this family share two conserved motifs: the HxxxD catalytic motif located in the middle of the protein sequence, and the C-terminal DFGWG motif involved in maintaining protein structural integrity (Galaz *et al.*, 2013; Morales-Quintara *et al.*, 2015). The first member of the acyl-CoA dependent BAHD acyl transferase superfamily to be crystallised was a vinorine synthase (an acetyl transferase) from *Rauvolfia serpentine*. From this structure, it was determined that the proteins in the BAHD family are composed of two equal sized domains connected through a large crossover loop (Ma *et al.*, 2005). To date, no crystal structure of a plant alcohol acyltransferase has been resolved, and as a result several groups have instead modelled these proteins in an attempt to identify: (i) the mode by which they catalyse the esterification reaction, (ii) the residues involved in composing the substrate binding pocket and how they determine the substrate specificity of an given AAT, and (iii) the role the individual residues that compose the conserved motifs play in catalysis and maintenance of solvent channel integrity (Morales-Quintara *et al.*, 2012; Morales-Quintara *et al.*, 2013; Galaz *et al.*, 2013; Morales-Quintara *et al.*, 2015; Navarro-Retamal *et al.*, 2016). As to date there is no publicly available crystal structure, or model, for the AAT protein from *A. chinensis*. Instead, here, a model was built for *EcAAT* using Modeller 9.18 software and three template crystal structures: a hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase from *Sorghum* (PDB: 4KE4), a hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyl transferase from *Coffea canephora* (PDB: 4G22), and the vinorine synthase from *R. serpentine* (PDB: 2BGH) (Ma *et al.*, 2005; Lallemand *et al.*, 2012; Walker *et al.*, 2013). The purpose of this model being that it would act as an aid in the rational protein engineering of the *EcAAT* protein for improved butyl octanoate product specificity. The templates used to build the *EcAAT* model were chosen based on their sequence similarity (33.25%, 32.9%, and 21.2%, respectively) and sequence coverage to the *EcAAT* protein. Of the 5 models produced through the modeller 9.18 software, the quality of the 3D protein structure of each model was assessed using a Ramachandran plot analysis (using RAMPAGE software). A Ramachandran plot assesses the backbone dihedral angles of each residue in the model and determines whether they are

favourable or unfavourable (Ho and Brasseur, 2005). From this analysis, the model with the highest Ramachandran value, which had 98% of residues in a favourable/allowed region, was chosen. This model can be seen in Figure 4.13 alongside the amino acid sequence for *EcAAT*. Both the sequence and model have been colour coded to identify protein domains I and II (colours blue and purple, respectively), the loop linker region (orange), the HxxxD catalytic motif (yellow), and the DFGWG structural motif (red).

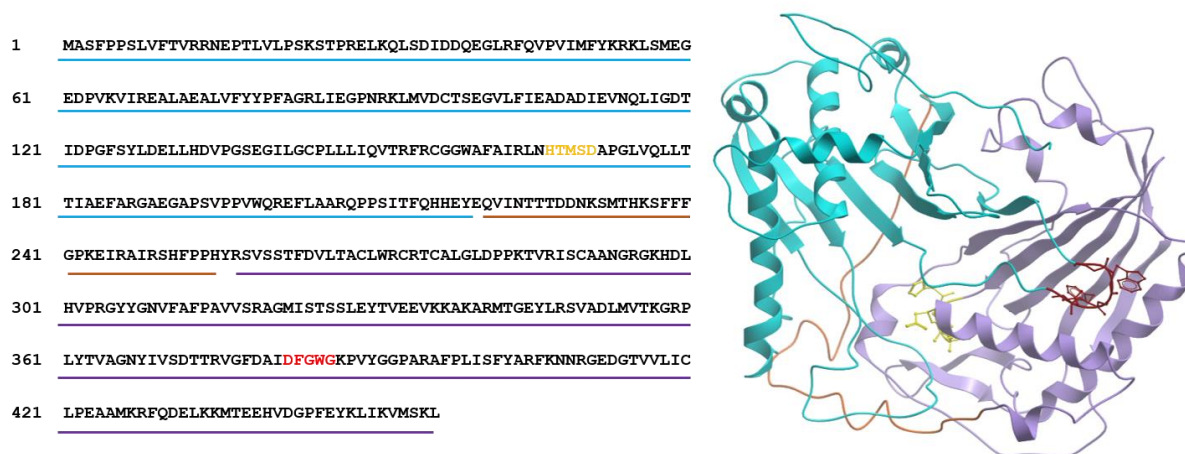


Figure 4. 13 Amino acid sequence (left) and protein model of *EcAAT* (right). The model was constructed using Modeller 9.18 software (www.salilab.org/modeller) with three template crystal structures (PMDDB codes: 4KEC, 4G22, and 2BGH2). Domain I = blue, cross over loop = orange, domain II = purple, HXXXD catalytic motif = yellow, and DFGWG structural motif = red. The residues in the protein sequence (left) corresponding to each colour coded region is underlined in its respective colour.

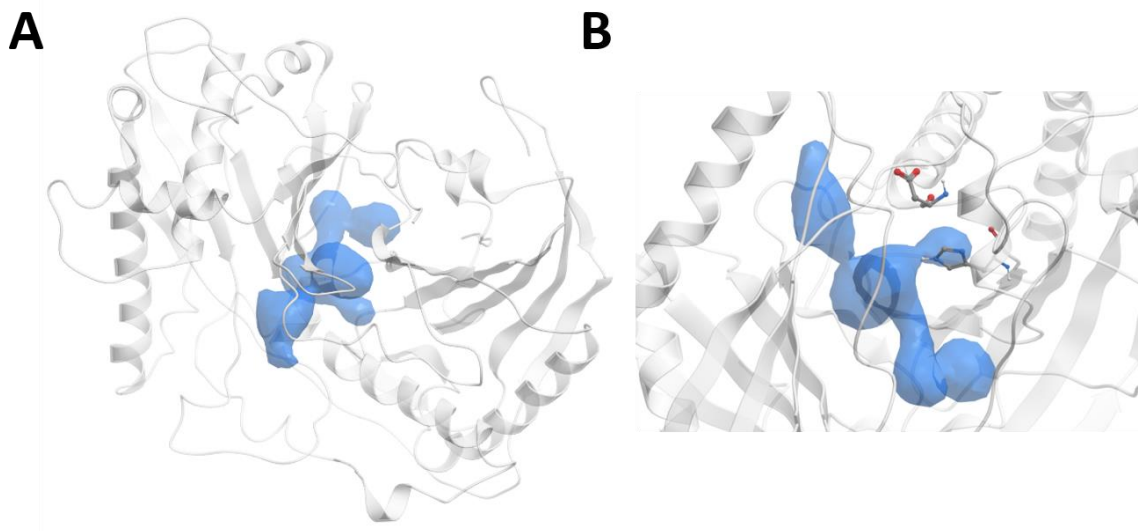


Figure 4. 14 Predicted solvent pocket of the modelled *EcAAT* enzyme. **A** shows the pocket in relation to the whole protein and **B** shows a close up of the pocket and its relation to the Histidine and Aspartic acid residues that are present in the catalytic motif. The solvent pocket was modelled using the Internal Coordinate Mechanics (ICM) program from www.molsoft.com.

Previous modelling work done for AAT enzymes from fruit have identified that both the alcohol and acyl-CoA substrate enter through a solvent channel located in the centre of the protein to access the catalytic residues (Morales-Quintara *et al.*, 2012; Galaz *et al.*, 2013; Navarro-Retamal *et al.*, 2016). Thus, this solvent channel into which the alcohol and acid substrates dock for esterification was further modelled for the *EcAAT* protein. From this, the residues that potentially compose this substrate binding pocket could be identified, facilitating the identification of target residues for site directed mutagenesis for increased substrate specificity for octanol-CoA. Morales-Quintara *et al.*, (2012) found that the binding pockets of two AAT enzymes from two different cultivars of papaya varied greatly in the size, corresponding with their respective substrate preferences for large and small acyl-CoA substrates. To model the solvent channel of *EcAAT*, ICMPOCKETFINDER software was used (Abagyan *et al.*, 1994; www.molsoft.com), which utilises only the protein 3D structure to predict cavities and clefts. Using this software, three potential solvent pockets were identified (data not shown), however only one was associated with the catalytic domain. Figure 4.14 shows this solvent pocket in the context of the entire protein model (panel A), and in the context of the catalytic HxxxD motif

(panel B). In panel B, it can be seen that the side chains of the histidine and aspartic acid residues of the catalytic motif that are directly involved in the esterification reaction are oriented into the solvent pocket, consistent with other models, and their role in catalysis (Morales-Quintara *et al.*, 2012; Galaz *et al.*, 2013). Following this, simulations using ICM docking software were performed (www.molsoft.com) to identify from which direction the alcohol and acyl substrates enter the solvent pocket docking. These simulations consider electrostatic and van der Waals forces as the main inputs (Galaz *et al.*, 2013). From this it was identified that the octanoyl-CoA substrate probably enters the solvent pocket through the bottom channel shown in panel B of Figure 4.14. This further narrowed down the number of residues that might influence *EcAAT* substrate specificity for the acyl chain length. Based on this evidence, each of these residues that compose this bottom channel was sequentially mutated to the corresponding residue(s) present at that location in other AAT enzymes, and the impact of each mutation on the solvent channel was assessed (a line up of all AATs assessed is found in Supplementary Figure 7). This was done as an attempt to prevent wholly unfavourable mutations with the presumption that should a given residue be present at that location in another AAT, that a mutation to that residue in the *EcAAT* protein is less likely to result in complete abolishment of activity. Where more than one residue was present at a given location in different AAT enzymes, each permutation was computationally assessed. The purpose of this work was to identify possible point mutations that would increase the size of the binding pocket channel into which the acyl-CoA chain enters. The rationale for this being that a larger channel might improve the ability of the larger octanoic chain length to access the catalytic residues. This represents a cursory approach to rational engineering of the *EcAAT* enzyme. In principle, by mutating the relevant residues to those present at that location in other functional AAT enzymes, the chances of making a mutation that decreased enzyme activity were minimized. From this, five point mutations were identified that favourably increased the size of the solvent channel, they were: M94F, S99G, L178F, F185I, and F313V. For the most part, these substitutions were from a hydrophobic residue to another hydrophobic residue. The exception being S99G where serine was substituted for glycine. Interestingly, glycine appeared to be highly conserved at this position in other AATs (Supplementary Figure 7). Additionally, if a mutation to known residues at the same location in other AATs did not increase the size of the solvent channel, the effect of mutating that residue to any alternative amino acid was assessed with regards to solvent channel size. With an arbitrary cut-off of a 7.5% increase in pocket size, two further potentially favourable mutations were identified: L42A and R248S. While neither of these substitutions are

present in other AAT enzymes, their effect on solvent channel size outweighed the risk of loss of activity for this preliminary work.

Each of these *EcAAT* point mutants were made through overlap PCR, and then cloned into a pET21a plasmid between restriction sites *Bam*HI and *Xho*I. The presence of each point mutation was confirmed through gene sequencing by GATC Biotech (Germany). Figure 4.15 shows the proportion of butyl butyrate, -hexanoate and -octanoate produced by *E. coli* C43 (DE3) expressing each of the point mutated *EcAAT* genes, as well as the wild type *EcAAT*. *EcAAT* expression was carried out as described in section 4.2.1, including substrate feeding. Four of these mutations – L42A, M94F, F185I, and R248S – severely reduced the rate of butyl ester production. Each of these mutants produced only butyl butyrate at significantly reduced titres compared to the wild type *EcAAT*, suggesting that they had either reduced the activity or production of active enzyme. The F313V mutation, interestingly, produced nearly 60% more butyl butyrate and 35% less butyl hexanoate than the wild type *EcAAT*, indicating it may have increased the protein's substrate specificity for the shorter butyrate chain length. Of the seven point mutations made, only two, S99G and L178F, resulted in a significant improvement in butyl octanoate production, with a 620% and 475% increase, respectively. In both cases, the corresponding residue to which the native residue was changed is highly conserved amongst other AAT enzymes. Not only did these mutations have an impact on final butyl octanoate titres, but they both also marginally increased the final titres of butyl hexanoate compared to the wild type *EcAAT* (though this increase was only significant for the S99G mutant, $p=0.0139$), while having no significant impact on the amount of butyl butyrate produced. This suggests that both the S99G and L178F mutations may favourably improve activity towards the longer hexanoyl-CoA and octanoyl-CoA chain lengths by increasing solvent channel size. While both of these mutants produced similar final butyl butyrate titres as the wild type *EcAAT*, this is understandable as a larger solvent channel would not preclude the binding of smaller substrates.

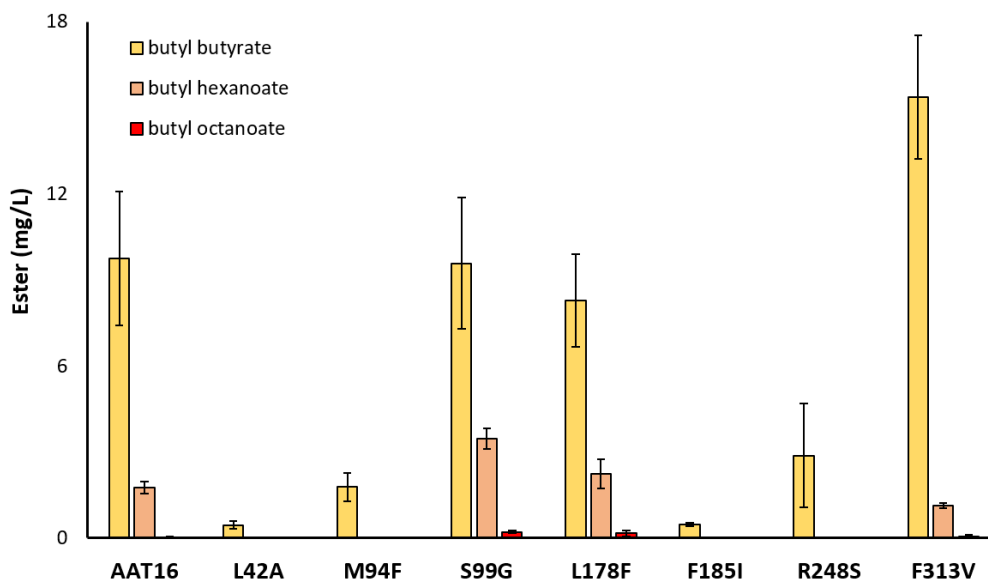


Figure 4. 15 Proportion of butyl butyrate, -hexanoate, and -octanoate present in the supernatant of *E. coli* cultures expressing the wild type *EcAAT* protein and seven point mutation *EcAAT* variants. The first letter represents the original residue before mutation, the number represents the location of the residue in the protein (where 1 would be the starting methionine), and the second letter represents the new residue present at that position in the mutant. L = leucine, A = alanine, M = methionine, F = phenylalanine, S = serine, G = glycine, I = isoleucine, R = arginine, V = valine. Data are the mean \pm standard deviation of three biological replicates.

To further determine whether these two individual point mutations were synergistic, a double *EcAAT* mutant of both S99G and L178F was created, cloned into pET21a, and transformed into *E. coli* strain C43 (DE3). Cultures of the double S99G L178F mutant were expressed alongside cultures of the single S99G and L178F mutants as well as the wildtype *EcAAT*, as described previously, for comparison. Final butyl ester profiles for each are shown in Figure 4.16. Here, it was found that while the double mutant appeared to produce more butyl octanoate than the next highest producer, the S99G mutant, this is not statistically significant ($p = 0.344$). Therefore, there appears to be no additive or synergistic effect in combining the S99G and L178F mutations. Unsurprisingly, none of these mutants increase substrate specificity towards longer acyl-CoA chain lengths as an increase in butyl octanoate production is not paired with a decrease in either butyl butyrate or -hexanoate production. By increasing the size of the solvent channel, the shorter chain esters are not precluded from accessing the active site (although the reverse may be true, vis F313V, Figure 4.15. Instead, to minimize the production of butyl

butyrate and hexanoate, it may be more effective to engineer endogenous *E. coli* fatty acid metabolism to decrease the availability of these shorter acid chain lengths.

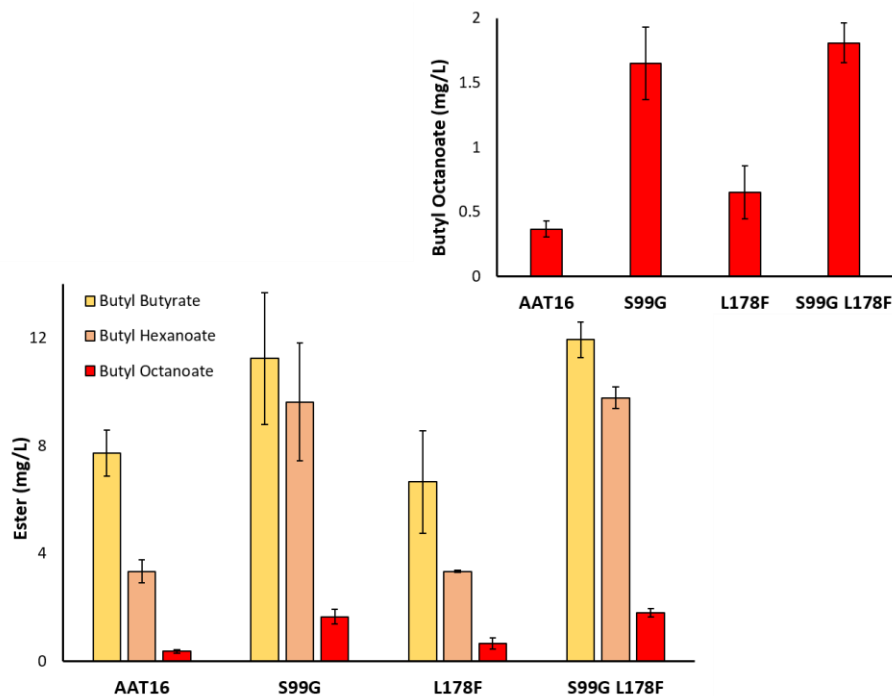


Figure 4. 16 Proportion of butyl butyrate, -hexanoate, and -octanoate present in the supernatant of *E. coli* cultures expressing the wild type *EcAAT* protein, the S99G single mutant, L178F single mutant, and the S99G L178F double mutant. The inset above shows the proportion of butyl octanoate produced by each of these four proteins. Data are the mean \pm standard deviation of three biological replicates.

4.2.4.2 Metabolic engineering

An alternative strategy to improve production of butyl octanoate over other chain acyl lengths would be to engineer the metabolism of *E. coli* to increase the intracellular availability of the octanoyl-CoA substrate, and decrease the availability of the competing butyryl and hexanoyl-CoA substrates. This strategy would involve the manipulation of *E. coli* fatty acid synthesis and

degradation and could be employed on its own, or in combination with protein engineering of the *EcAAT* protein for improved substrate specificity towards the octanoyl-CoA substrate. As well, this strategy could be used in conjunction with octanoic acid feeding to maximize intracellular availability of this substrate. Fatty acid synthesis (FAS) and degradation (FAD) are two highly regulated processes of lipid metabolism in *E. coli* and involves the *de novo* synthesis of fatty acids from acetyl-CoA and the degradation of fatty acids to acetyl-CoA, respectively (Clark and Cronan, 2005; Lennen *et al.*, 2012; Janßen *et al.*, 2014). Through fatty acid synthesis, intracellular acetyl-CoA, obtained from a number of carbon sources, is converted in several enzymatic steps to acetoacetyl-ACP, the first molecule in the fatty acid elongation cycle. This substrate is then further extended in chain length by two carbons for every cycle with the addition of an acetate (donated from malonyl-ACP) to the growing acyl-ACP chain through the activity of five enzymes, FabG, FabZ, FabI, FabF, and FabB (Figure 4.17 right). This process primarily produces C16:0, C16:1, and C18:1 acyl-ACPs which are then further incorporated into phospholipids, fatty aldehydes, triglycerides, etc., or instead may be hydrolysed to produce a free fatty acid (Fujita *et al.*, 2007; Lennen *et al.*, 2012). The production of a free fatty acid, specifically, from an acyl-ACP substrate occurs via the enzymatic removal of the ACP unit by a thioesterase enzyme (TE). Thioesterases are a member of the hydrolase family and are responsible for the cleavage of the acyl-ACP thioester to a free fatty acid and ACP-SH (Figure 4.17; Hunt *et al.*, 2002). They often possess distinct substrate specificity for the chain length and saturation of the acyl-ACP substrate, and ultimately control the composition of free fatty acids in the cell (Salas and Ohlrogge, 2002). Unlike in FAS, during FAD, a free fatty acid is first esterified to a CoA-SH to produce a fatty acyl-CoA. Doing so not only primes the fatty acid to enter the β -oxidation cycle for degradation, but acyl-CoAs may also act as substrate for the further production of wax esters, ketones, phospholipids, etc. (Clark and Cronan, 2005; Fujita *et al.*, 2007; Lennen *et al.*, 2012). Should the acyl-CoA enter the β -oxidation pathway, it is iteratively shortened in chain length by two carbons for every one cycle (Figure 4.17) to produce acetyl-CoA that may be further utilized other metabolic processes.

Many groups have worked towards increasing the free fatty acid production of *E. coli* through manipulation of the FAS and FAD pathways; often as a means for biofuel production (Lu *et al.*, 2008; Lennen *et al.*, 2010; Lui *et al.*, 2010; Dellomonaco *et al.*, 2011; Jeon *et al.*, 2011; Lennen *et al.*, 2011; Zhang *et al.*, 2011; Zhang *et al.*, 2012; Youngquist *et al.*, 2012). Typically, this process has involved three general steps: upregulation of FAS, expression of a TE enzyme that possesses specificity for the desired free fatty acid(s), and downregulation of FAD. Unlike these

groups, however, this work is interested in increasing the availability of a specific acyl-CoA – octanoyl- CoA. Thus far, octanoic acid has been exogenously fed into cultures to increase the intracellular concentration of this acid chain length, which has been moderately successful. Exogenously fed fatty acids are converted directly to acyl-CoAs upon movement into the cytosol (as there is no mechanism in *E. coli* for the conversion of exogenously fed fatty acids to acyl-ACP). Thus, exogenously fed octanoic acid is converted to directly to octanoyl-CoA intracellularly, the desired acyl chain length for butyl ester production. However, it was found that upon exogenous octanoic acid and butanol feeding

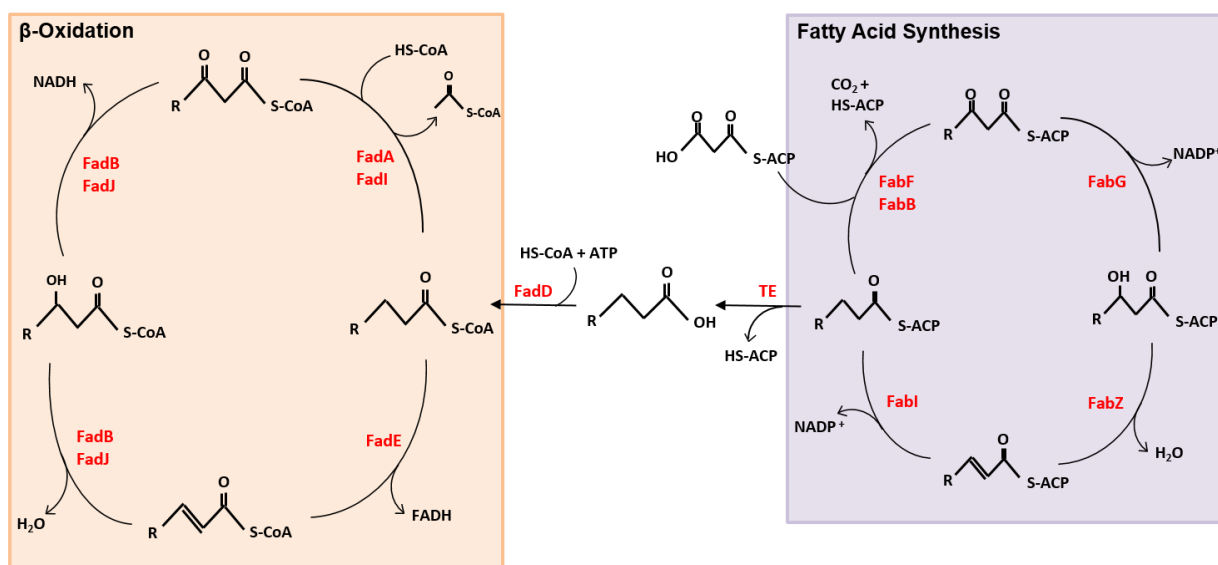


Figure 4. 17 Bacterial metabolic pathways for the synthesis of free fatty acids (right) and β-oxidation for the breakdown of free fatty acids (left). Fab = Fatty Acid Biosynthesis, Fad = Fatty Acid Degradation. Modified from Lennen *et al.*, (2012).

into cultures expressing *EcaAT*, that significantly more butyl hexanoate and -butyrate were produced than butyl octanoate. Also, it was noted that *E. coli* C43 (DE3) cultures expressing *EcaAT* in which only butanol, and no acid, was exogenously fed produced only trace amounts of butyl butyrate and butyl hexanoate and no butyl octanoate (data not shown). Together, this suggests that in *E. coli* cultures exogenously fed octanoic acid, that it is being oxidized by the

FAD pathway after its conversion to octanoyl-CoA in the cell to the shorter C6:0 and C4:0-CoA chain lengths, and that these substrates are then preferentially being esterified over the octanoyl-CoA by the *EcAAT* enzyme. As these shorter chain butyl esters are toxic to *E. coli*, a strategy similar to that employed by other groups working towards engineering increased free fatty acid production in *E. coli* was used here to engineer an increase in octanoyl-CoA abundance, and minimize its degradation to the shorter chain hexanoyl and butyryl-CoAs in *E. coli*.

First, the possibility of increasing the proportion of octanoyl-CoA over hexanoyl- and butyryl-CoA upon exogenous octanoic acid feeding by downregulating FAD was investigated. To increase free fatty acid abundance in *E. coli*, many groups have knocking out the *FadD* gene, which is an acetyl-CoA synthase responsible for converting a free fatty acid to an acyl-CoA (Yoo *et al.*, 2001). However, doing this would be disadvantageous for the present work as acyl-CoA is the substrate for AAT enzymes. Therefore, *FadD* activity is required to convert exogenously fed – or endogenously produced – octanoic acid to octanoyl-CoA. Instead, an *E. coli* *FadE* gene knockout strain (JW5020) from the CGSC (Coli Genetic Stock Center; Baba *et al.*, 2006) was obtained. *FadE* is the enzyme responsible for the second reaction step in β -oxidation, which is the conversion of an acyl-CoA to an enoyl-CoA (Figure 4.17). This would mean that in a *FadE* knockout strain, exogenously fed octanoic acid should continue to be converted to octanoyl-CoA by the action of *FadD*, but not further degraded by β -oxidation to the hexanoyl and butyryl-CoA chain lengths. Several groups have used Δ *FadE* strains in the past to successfully increase the production of a number of valuable acyl-CoA derivative products (Steen *et al.*, 2010; Yu *et al.*, 2011). The JW5020 *E. coli* strain was transformed with pFRANK and cultured for butyl ester production as described previously in section 4.2.1, including butanol and octanoic acid feeding. Unexpectedly, this strain produced no butyl ester of any chain length, however it was observed that at the point of analysis at 18 hours, that all octanoic acid had been consumed (data not shown). This was interesting as it has previously been observed in cultures of wildtype C43 (DE3) *E. coli* exogenously fed octanoic acid that at the point of analysis at 18 hours that a significant amount of octanoic acid remained (data not shown). As other groups had successfully expressed a long chain alcohol acyltransferase (also called a wax synthase) in a Δ *FadE* *E. coli* knockout strain to produce high titres of long chain FAEs (fatty acid ethyl esters) (Steen *et al.*, 2010), it was initially suspected that the JW5020 strain acquired was not a true *FadE* knockout. Therefore, to confirm the presence of the gene knockout in the JW5020 strain, the genomic region in which the *FadE* gene is located was amplified by PCR in wildtype *E. coli* strain C43 (DE3) and strain JW5020. From this, it was

found that strain JW5020 was, in fact, a true *FadE* knockout, as the DNA band size amplified corresponds to the expected size of the kanamycin resistance gene, and not the *FadE* gene (as seen in the C43 (DE3) strain) (Figure 4.18). Initially, an explanation as to why the *EcAAT* produces no butyl ester in the JW5020 background is unclear, especially considering that the total consumption of exogenously fed octanoic acid in culture was observed. It may be the case that the octanoic acid was being consumed by other lipid synthesizing pathways that are up regulated to compensate for the decreased activity of β -oxidation in this *FadE* knockout strain. Acyl-CoA substrates, as mentioned above, can also be converted to a number of other lipid product *in vivo*; such as wax esters, triglycerides, ketones, phospholipids, etc. It may be the case that one or more of these pathways has been upregulated to compensate for the decrease in FAD, and that these endogenous pathways are outcompeting the *EcAAT* for substrate. To preliminarily investigate this, the fatty acid profiles of *E. coli* strains C43 (DE3) and JW5020

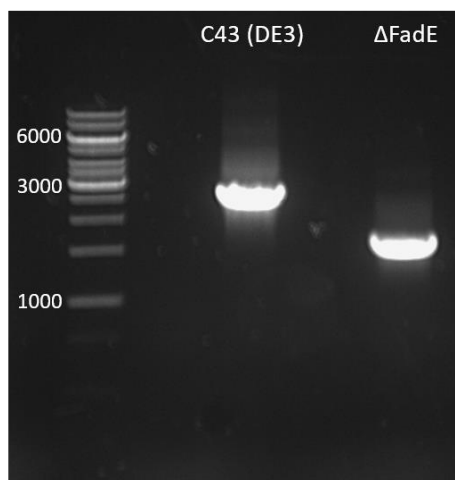


Figure 4. 18 Agarose gel showing the PCR amplification over the genomic region containing the *FadE* gene for both wild type *E. coli* C43 (DE3) strain and JW5020 ($\Delta FadE$) knock-out strain. Primers annealed 150 bp upstream and downstream of the *FadE* gene.

exogenously fed 2.5 mM octanoic acid were compared. Cultures were incubated for 18 hours at 20°C from the point of octanoic acid addition. This analysis was of all free and bound

intracellular fatty acids, and thus included free acids as well as those present in esters, triglycerides, phospholipids etc. Figure 4.19 demonstrates the proportion of each fatty acid present in C43 (DE3) and JW5020. Here it can be seen that the JW5020 strain possessed more than double the amount of octanoic acid compared to the C43 (DE3) strain of *E. coli*. Unlike in the wildtype C43 (DE3) *E. coli* strain, where the octanoic acid is both entering β -oxidation and being converted to other lipid compounds, the JW5020 strain is unable to breakdown this acid, accounting for its increased abundance. This, paired with the observations that no free octanoic acid remained in cultures of *E. coli* strain JW5020 expressing *EcAAT* that had been exogenously fed this acid for the purposes of butyl ester formation, suggested, rather, that instead it is being incorporated into other lipid compounds. In both the C43 (DE3) and JW5020 *E. coli* strains, the amount of detected acid of chain lengths below C8:0 were too small to reliably quantify. Interestingly, the amount of each individual fatty acid quantified from *E. coli* strain JW5020 was either equal to or smaller than the respective amount quantified from strain C43 (DE3). This was unexpected as downregulation of β -oxidation should affect abundance of all fatty acids in this knockout strain. Unfortunately, upon reflection of this work, the explanation for the lack of *EcAAT* activity in this Δ *FadE* genetic background became clear, and is a result of an unremarkable and trivial error. The parent strain of JW5020 is *E. coli* K-12, a strain that does not possess the gene that codes for T7 RNA polymerase. Thus, transcription of the *EcAAT* gene from pFRANK, which is under the control of a T7 RNA polymerase promoter, is not possible in the JW5020 strain. While this is an unfortunate error, it does mean that the use of a Δ *FadE* *E. coli* strain that does contain the T7 RNA polymerase gene – such as C43 (DE3) – may still be a valid strategy for increasing the intracellular abundance of octanoyl-CoA by minimizing its degradation to shorter acyl-CoAs by β -oxidation; and thus improving butyl octanoate product specificity via *EcAAT* esterification. The next approach for increasing the abundance of available octanoyl-CoA in the cell was to express a thioesterase enzyme that has substrate specificity for hydrolysing octanoyl-ACP to octanoic acid. Thioesterase enzymes typically possess activity for either CoA or ACP linked substrates, though some are able to work on both. This work required the employment of a thioesterase that is specific for hydrolysing only the octanoyl-ACP produced through *de novo* fatty acid synthesis – as a thioesterase with any

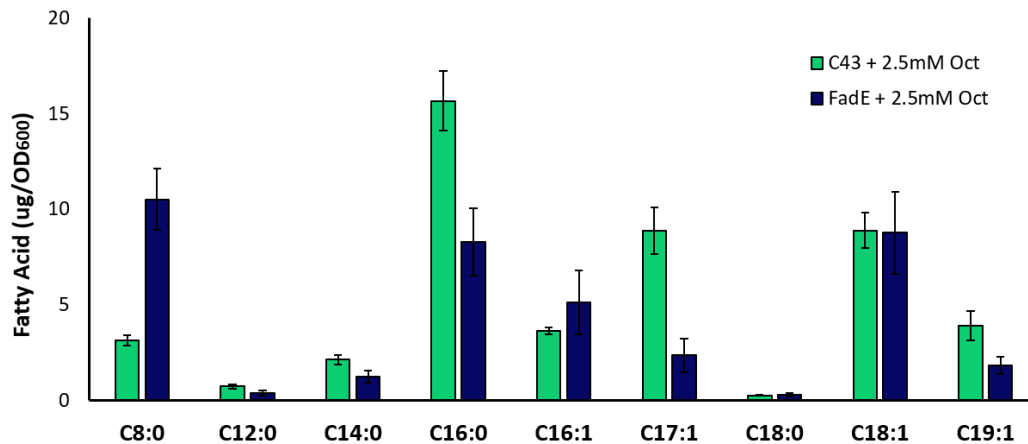


Figure 4. 19 Analysis of the total internal lipid content of *E. coli* strains C43 (DE3) and JW5020 (Δ FadE). Cultures were exogenously fed 2.5 mM octanoic acid and grown for 24 hours before analysis. Data are the mean \pm standard deviation of three replicates.

activity for hydrolysing octanoyl-CoA would be detrimental to this system as it is the direct substrate of the *EcAAT* enzyme. A number of thioesterases from plants have been characterized, and one specifically from *Cuphea palustris*, named FATB1, demonstrates nearly exclusive substrate specificity for hydrolysing a C8:0-ACP to octanoic acid (Dehesh *et al.*, 1996; Jing *et al.*, 2011). Ultimately, the expression of *CpFATB1* (where *Cp* stands for *Cuphea palustris*) would be most beneficial in an *E. coli* background where β -oxidation is also downregulated (such as the Δ FadE trialled above), as it would establish a system where free octanoic acid abundance is increased via the TE activity and its degradation to other shorter acid chain lengths is decreased via β -oxidation perturbation. This has been done previously by Torella *et al.*, (2013) by the expression of a thioesterase in an *E. coli* Δ FadD strain, where free fatty acid titres were improved over a wildtype *E. coli* strain expressing the same thioesterase. As the reason for the lack of butyl ester production in the JW5020 *E. coli* strain via *EcAAT* expression from pFRANK had not been elucidated at this point, expression of the C8:0-ACP specific FATB1 thioesterase from *C. palustris* was done in both the wild type C43 (DE3) *E. coli* strain and the JW5020 Δ FadE knockout strain – where the JW5020 strain was used as an example of a β -oxidation hindered strain. Additionally, the potential benefit of supplementing cultures with the antibiotic cerulenin was investigated. The mode of action of cerulenin is to target fatty acid synthesis enzymes FabB and FabF, thus inhibiting fatty acyl-ACP elongation but not the initial condensation reaction of FabH that enter acetoacetyl-ACP into FAS (Kawaguchi *et al.*, 1982;

Torella *et al.*, 2013). The result of this is an accumulation of medium chain acyl-ACP *in vivo*; which is most likely a consequence of both the decreased elongation of medium chain acyl-ACPs to long chain acyl-ACPs, as well as the resulting relief in feedback inhibition of FabH due to the decrease in long chain acyl-ACP abundance (Jiang and Cronan, 1994; Heath and Rock, 1996; Torella *et al.*, 2013). Thus, the *CpFATB1* sequence was codon optimized to the genome of *E. coli*, synthesized as a gene string by GeneArt, and subsequently cloned into pET21a between restriction sites *Bam*HI and *Xho*I. pET21a::*CpFATB1* was then transformed into both *E. coli* strains C43 (DE3) and JW5020. Cultures of C43 (DE3) and JW5020 harbouring pET21a::*CpFATB1* were incubated in M9 + 1% glycerol media and induced with 0.4 mM IPTG before being incubated at 30°C for 24 hours. To those cultures in which cerulenin was added, this was done at the point of induction. Figure 4.20 shows the abundance of total octanoic acid present in the cell pellet fraction both *E. coli* strains with or without *CpFATB1* expression or the addition of cerulenin. Here it can be seen that heterologous expression of this thioesterase increased the intracellular abundance of octanoic acid in both the *E. coli* C43 (DE3) and JW5020 strains by 2-fold and 5-fold, respectively, over their individual controls. As well, media supplementation with cerulenin further increased the titres of octanoic acid achieved by both *E. coli* strains, with a 3.8-fold increase over the control for strain C43 (DE3) expressing *CpFATB1*, and a 9.5-fold increase over the control for strain JW5020 expressing *CpFATB1* (Figure 4.20). The higher abundance of octanoic acid produced by the cultures of JW5020 harbouring pET21a::*CpFATB1* compared to the JW5020 control suggests that *CpFATB1* is being expressed to some degree regardless of the lack of T7 RNA polymerase in this strain. The accumulation of all other fatty acids was not significantly different between these two strains in each condition trialled (data not shown). Overall, *CpFATB1* thioesterase expression in the Δ *FadE* background resulted in significantly higher octanoic acid titres compared to the C43 (DE3) wildtype strain, suggesting that the use of an *E. coli* Δ *FadE* strain that possesses T7 RNA polymerase would be highly beneficial to this strategy for increasing intracellular octanoyl-CoA abundance. While the expression of *CpFATB1* successfully increased the intracellular abundance of octanoic acid in both *E. coli* strains C43 (DE3) and JW5020, this is a measurement of total octanoic acid in the cell, and not necessarily octanoyl-CoA – our desired product. The enzyme responsible for the conversion free fatty acid to acyl-CoA is an acyl-CoA synthetase enzyme named FadD, which activates these substrates for β -oxidation (Yoo *et al.*, 2001). As such, it is often a knockout target for metabolic engineering aimed at increasing free fatty acid production in *E. coli* (Lu *et*

al., 2008; Steen *et al.*, 2010; Lennen *et al.*, 2011; Zhang *et al.*, 2012). Conversely, however, it has also been demonstrated that overexpression of the native *E. coli* FadD in conjunction with

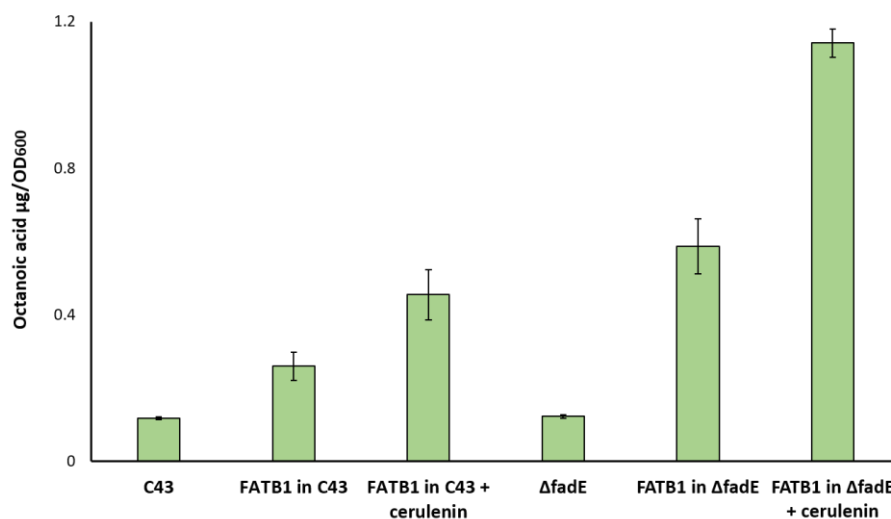


Figure 4. 20 Total internal octanoic acid content of *E. coli* strains C43 (DE3) and JW5020 (Δ FadE) alone, heterologously expressing FATB1 from *C. palustris*, or heterologously expressing FATB1 in the presence of the antibiotic cerulenin to retard fatty acid elongation. Cultures were grown in M9 media + 1% glycerol and induced with IPTG at an OD₆₀₀ of 0.8. Cerulenin addition occurred at the point of induction. Cultures were grown for 24 hours before lipid analysis. Data are the mean \pm standard deviation of three biological replicates.

heterologous thioesterase expression improves the production of acyl-CoA derived products—such as FAEEs and fatty alcohols – over thioesterase expressing *E. coli* strains where there was no FadD overexpression (Steen *et al.*, 2010). This suggests that FadD overexpression increases the conversion of free fatty acids to acyl-CoAs. As such, it was hypothesized that co-expression of the heterologous CpFATB1 and homologous FadD in *E. coli* would increase the abundance of available octanoyl-CoA in the cell, which could then act as a substrate for butyl octanoate production by the EcAAT enzyme. Therefore, plasmid pVICTOR 2.0 was further modified by inserting the CpFATB1 gene and *E. coli* FadD gene downstream of the Fdh gene to create a new plasmid named pSHELLEY (Figure 4.21). Ideally, this pSHELLEY plasmid would be co-

transformed with the pEL11 plasmid into an *E. coli* derivative strain of JCL166 which also possesses a *FadE* gene knockout for perturbation of β -oxidation. In theory, this strain would be capable of (i) endogenous production of butanol, (ii) increased production of octanoyl-CoA with minimal degradation of this acyl chain length, and (iii) conversion both of these substrates to butyl octanoate via *EcAAT* esterification. Additionally, this strain could be exogenously fed octanoic acid should the endogenously produced titres be limiting. However, for this to be possible, resolution of the overhanging problem of *Ter-EcAAT* co-expression would be required. Until this problem is resolved, quantification of the impact *CpFATB1* and *FadD* overexpression have on the total amount/composition of butyl esters produced by *E. coli* expressing pSHELLEY is not possible.

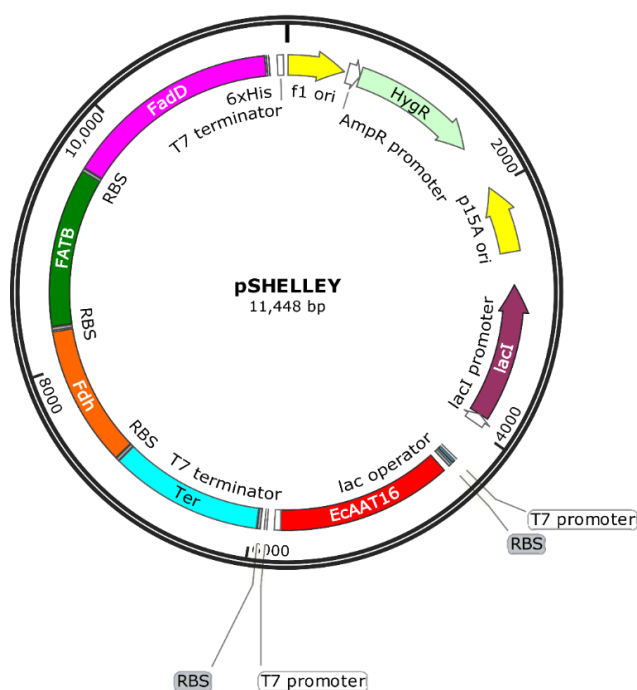


Figure 4. 21 Plasmid map of pSHELLEY, a derivative of pVICTOR2.0. *FATB* from *C. palustris* was cloned between restriction sites *SalI* and *SacI*, and *FadD* from *E. coli* was cloned between restriction sites *SacI* and *NotI*.

4.3 Conclusion

In this work, the detoxification of endogenously produced butanol via incorporation into the non-toxic butyl octanoate ester in *E. coli* by an AAT enzyme from *A. chinensis* was investigated. Supplementation of *E. coli* cultures heterologously expressing the AAT with both butanol and octanoic acid resulted in a mix of butyl octanoate, -hexanoate, and -butyrate esters; the latter two of which were undesirable end-products as they are more toxic to *E. coli* than butanol. Activity of the AAT enzyme in an *E. coli* strain that endogenously produces butanol resulted in very low titres of butyl ester, and further investigation identified that AAT expression in this strain is reduced due to co-expressed with a *Ter* gene that encodes for a protein that carries out an intermediate step in the butanol production pathway. Efforts to improve AAT substrate specificity towards the octanoic acid substrate chain length via protein engineering identified two point mutations that increase butyl octanoate production five times over the wildtype enzyme, but did not reduce production of butyl hexanoate or -butyrate. In this chapter, efforts to engineer an *E. coli* strain to increase its endogenous accumulation of the desired octanoyl-CoA substrate, and to reduce the degradation of octanoyl-CoA to the shorter chain hexanoyl-CoA and butyryl-CoA substrates, was begun, but not finished. However, should a compatible $\Delta FadE$ knockout *E. coli* strain be created, it may be possible to improve butyl octanoate titres via AAT activity in this engineered strain.

Ultimately, however, developing a strain of *E. coli* that incorporates endogenously produced butanol into butyl octanoate effectively enough for detoxification would require a significant protein and strain engineering. Incorporation of butanol into butyl ester as a general strategy for detoxification warrants further investigation, however butyl octanoate may not be a practical end-product for butanol detoxification due to the low substrate specificity of the AAT enzyme and the low AAT gene expression when co-expressed with the *Ter* gene required for butanol production.

5. Esterification as a strategy for reducing toxicity of geraniol and improving both yield and recovery from *E. coli* engineered for monoterpene production

5.1 Introduction

Geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) is an acyclic monoterpene alcohol found in the essential oils of plants such as lemongrass, rose, and geranium (Talapatra, S.K., and Talapatra, B., 2015). Due to its distinctly sweet and floral aroma, it has played a significant role in the flavour, fragrance, and cosmetic industries. More recently, however, several pharmaceutical applications for geraniol have been suggested, including use as an anti-cancer, anti-inflammatory, and pain relief agent. Additionally, geraniol has also been proposed as a promising gasoline alternative, superior to other biofuels such as ethanol, by virtue of its low volatility, low hygroscopicity and high energy density (Peralta-Yahya and Keasling, 2010; Guimarães *et al.*, 2013; de Cássia da Silveira e Sá *et al.*, 2013; Cho *et al.*, 2016; Liu *et al.*, 2016). As a consequence, the worldwide demand for geraniol has surpassed 1000 metric tons/year (Lapczynski, 2008). Currently, the predominant methods for obtaining geraniol include extraction from plant material and chemical synthesis; both of which are costly and inefficient (Sell, 2003; Liu *et al.*, 2015). Therefore, there has been a growing interest in developing biotechnological methods for terpene production in a more sustainable and economical way (Ignea *et al.*, 2013). Over the last decade, systems metabolic engineering of microbial hosts, such as *Saccharomyces cerevisiae* and *E. coli*, has proven to be successful for the production of a number terpenes from renewable resources (Martin *et al.*, 2003; Leonard *et al.*, 2010; Lee *et al.*, 2012; Ding *et al.*, 2014).

Monoterpene biosynthesis is dependent on the condensation of two C₅ isoprene precursors, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), to produce geranyl pyrophosphate (GPP) – the universal building block to all monoterpenes. These C₅ precursors can be synthesized via two independent pathways: the mevalonate-dependent (MEV) pathway and the methylerythritol 4-phosphate (MEP) pathway. Prokaryotes, such as *E. coli* typically use a native MEP pathway (Martin *et al.*, 2003; Hunter, 2007; Zebec *et al.*, 2016).

Early efforts to develop *E. coli* as a platform for commercial terpene production focused both on improving the expression and regulation of this native MEP pathway, as well as heterologous expression of the non-native MEV pathway sourced from a number of host organisms (Farmer *et al.*, 2000; Huang *et al.*, 2001; Alper *et al.*, 2005; Martin *et al.*, 2003; Pitera *et al.*, 2007; Anthony *et al.*, 2009; Yoon *et al.*, 2009; Redding-Johansen *et al.*, 2011). In recent years, the iterative optimization of both the MEP and MEV pathways in *E. coli* for the production of various end point terpenes has been undertaken, with notable success in the production of several sesquiterpenes, such as artemisinic acid, bisabolene and farnesene (Tsuruta *et al.*, 2009; Peralta-Yahya *et al.*, 2011; Wang *et al.*, 2011). In contrast to the high microbial titres achieved for these sesquiterpenes, monoterpene production in *E. coli* has been significantly less successful, hindering industrial scale production.

Efforts to produce monoterpenes from engineered *E. coli* has proven to be challenging due to their high toxicity compared to the longer chain sesqui- and diterpenes, with typical titres remaining in the mg/L range. Several groups have developed *E. coli* strains for geraniol production, though, to date, the titres achieved have been too low to be economically viable (Shah *et al.*, 2013; Zhou *et al.*, 2014; Liu *et al.*, 2015; Liu *et al.*, 2016). The obstacles that impede high geraniol production are (i) its high microbial toxicity and (ii) product loss due to bioconversion to other monoterpenoids – which are themselves toxic to various degrees – (Trombetta *et al.*, 2005; Chen and Viljoen, 2010; Brennan *et al.*, 2012) by endogenous *E. coli* enzymes. Geraniol toxicity has been attributed to its amphiphilic nature, endowing it with the ability to interact with cell membranes and impact their integrity and permeability, as well as interact with intracellular components (Trombetta *et al.*, 2005; Brennan *et al.*, 2012). Shah *et al.*, (2013) demonstrated that geraniol exposure in *E. coli* causes DNA damage, adding further evidence that this compound has a multifaceted mode of toxicity. In addition to the problem of microbial toxicity, several groups have reported loss of geraniol in *E. coli* cultures due to biotransformation of the endogenously produced geraniol to other geranoids (geranial, nerol, neral) and monoterpenoids such as citronellol and linalool (Fisher *et al.*, 2013; Zhou *et al.*, 2014). This has been attributed to the presence of promiscuous endogenous *E. coli* enzymes that possess monoterpene alcohol modifying capability; one such identified enzyme is *yjgB*, a geraniol dehydrogenase that converts geraniol to geranial (Zhou *et al.*, 2014). Ultimately, the result of these biotransformations is a decrease in titre and purity of the desired end-product – geraniol.

Both of the above-mentioned obstacles impeding microbial monoterpene production need to be resolved in order to achieve industrially viable geraniol titres from *E. coli*. Typically, to address the former issue of toxicity, microbial cultures producing terpenes are grown in the presence of a secondary organic phase, the purpose of which is the *in situ* sequestration of the produced terpene to minimize its exposure to the microbial cells and alleviate toxicity (Brennan *et al.*, 2012). While this has been successful for hydrophobic sequi- and diterpenes that partition well into the organic phase, the amphiphilic nature of monoterpenes means they partition less efficiently and remain, in part, in the aqueous milieu where they can continue to interact with cellular activity. Further, this inefficient sequestration facilitates their exposure to promiscuous endogenous enzymes that are capable of modifying the desired monoterpene end-product. Ultimately, terpene hydrophobicity/phility appears to be a central factor in the success of sequi- and diterpene production from microbial culture, and the failure of monoterpene production. This would suggest that employing a strategy where the hydrophobicity of a monoterpene is increased would promote its sequestration into a second organic phase. Here it is hypothesized that the further conversion of geraniol to a more hydrophobic acetate ester may improve final titres and minimize microbial toxicity by promoting its extraction into the organic layer of a two-phase culture. The partial acetylation of monoterpenoids has been previously reported for geraniol and perillyl alcohol via the promiscuous activity of a chloramphenicol acetyltransferase (CAT) used as a resistance marker in the plasmids harbouring these heterologous pathways (Alonso-Gutierrez *et al.*, 2013; Liu *et al.*, 2016). However, a rational investigation into this esterification strategy for the improvement of monoterpene extraction in a biphasic culture has not been done. Here this strategy is explored by the deliberate conversion of geraniol to geranyl acetate *in vivo* through the heterologous activity of an alcohol acyltransferase (AAT) enzyme from *Rosa hybrida* that possesses distinct substrate specificity for the acetylation of geraniol.

In this work, the strategy for detoxification via esterification that was previously attempted for butanol production was applied to the toxic monoterpene, geraniol. To do this, three aims were outlined: (1) to engineer *E. coli* to produce geranyl acetate from glucose through the heterologous expression of the mevalonate pathway, a geraniol synthase (GES) from *Ocimum basilicum*, and an alcohol acyltransferase (AAT) from *R. hybrida* (Figure 5.1); (2) to optimize the production of geranyl acetate in flask culture; (3) to scale up production of geranyl acetate to 1.5 L bioreactor.

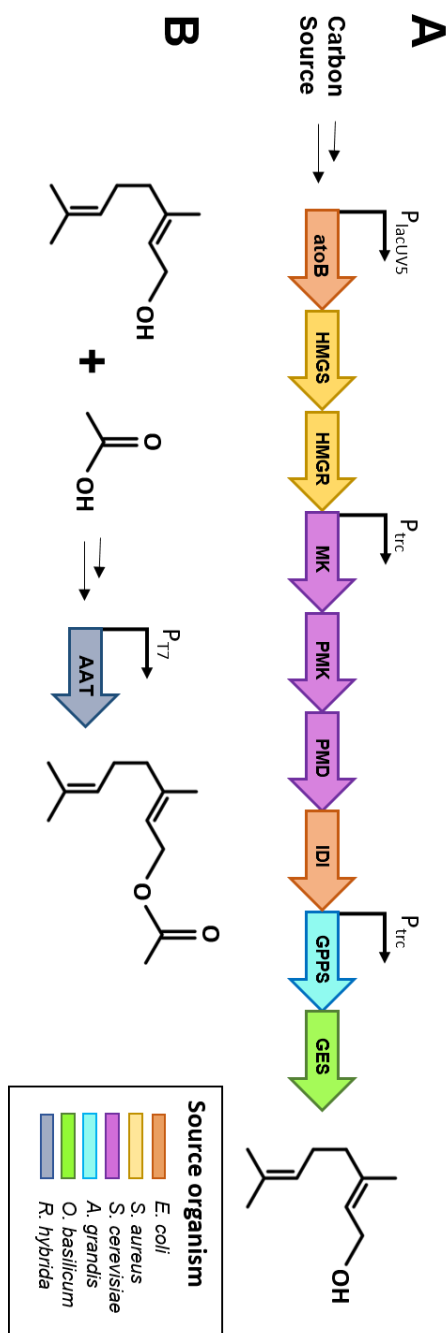


Figure 5. 1 Diagram of the two *E. coli* expression constructs used in this study harbouring (A) the pMIB13 plasmid harbouring a mevalonate pathway (MEV) leading towards the production of the monoterpene geraniol. Plasmid pMIB13 was modified from the limonene producing plasmid pJBEI-6410 (Alonso-Gutierrez *et al.*, 2013) by replacing the terminal terpene synthase with one specific for making geraniol (GES) from *O. basilicum*. (B) A second plasmid, pET28a::RhAAT, harbouring an alcohol acyltransferase (AAT) from *R. hybrida* with activity for esterifying geraniol and acetyl-CoA to produce geranyl acetate.

5.2 Results and Discussion

5.2.1 Geraniol and geranyl acetate production in *E. coli*

The previously described pJBEI-6410 plasmid (Alonso-Gutierrez *et al.*, 2013), harbouring an optimized MEV pathway with the requisite genes for limonene production was modified for the production of geraniol (Figure 5.1). This was done by replacing the terminal limonene synthase gene (*LS*) present in pJBEI-6410 with a geraniol synthase gene (*GES*) from *O. basilicum* – resulting in a modified plasmid that was named pMIB13. The *GES* enzyme from *O. basilicum* was chosen as the geraniol producing monoterpene synthase for this work as it has been well characterised in the literature. The full-length coding sequence of the *GES* gene was optimized to the genome of *E. coli*, including the N-terminal plastidial targeting sequence, which was not truncated as it had previously been demonstrated not to interfere with expression of the recombinant enzyme (Iijima *et al.*, 2004; Liu *et al.*, 2016; optimized *GES* sequence in Appendix). The replacement of the *LS* gene with the codon optimized *GES* gene was done using Gibson Assembly cloning, however as there was no available restriction site directly upstream of the *LS* gene in pJBEI-6410, the plasmid was instead cut at restriction sites *KpnI* and *BamHI* – the former site located 185 bp into the upstream Geranyl PyroPhosphate Synthase (*GPPS*) gene, and the later located directly downstream of the *LS* gene. A DNA fragment composed of the remaining 709 bp of the upstream *GPPS* gene followed by a *SalI* restriction site and the *GES* gene was flanked on either end with Gibson overhangs complementary to the vector sequence (primer sequences in Appendix) and inserted into pJBEI-6410 to reconstitute the *GPPS* gene and replace the limonene synthase with the desired geraniol synthase. The insertion of a *SalI* restriction site directly upstream of the *GES* gene was done to facilitate any future exchange of the terpene synthase. Figure 5.2 shows an agarose gel of the DNA digests of plasmid pMIB13 at either restriction sites *KpnI/BamHI* or *SalI/BamHI* to demonstrate the successful insertion of the *GES* gene and *SalI* restriction site, as well as the reconstitution of the *KpnI* restriction site. A full plasmid map of pMIB13 is shown in Supplementary Figure 8 of the Appendix.

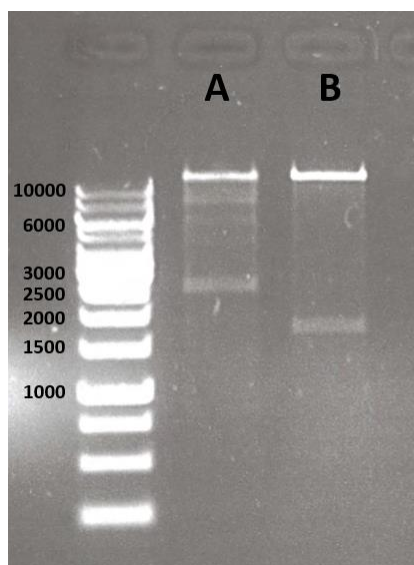


Figure 5. 2 Agarose gel showing the DNA double digests of pMIB13 with either restriction enzymes *KpnI/BamHI* (lane A) or *SalI/BamHI* (lane B). Lane A shows the 2400 bp fragment composed of the inserted *GES* gene with ~700 bp of the upstream *GPPS* gene. Lane B shows the 1700 bp fragment of the inserted *GES* gene, having been digested with the newly constituted *SalI* restriction site directly upstream of the inserted *GES* gene.

E. coli strain C43 (DE3) was then transformed with plasmid pMIB13 to create a geraniol producing *E. coli* strain named DLG2 (Methods Table 2.1). Strain DLG2 was cultured for 24 hours at 30°C after induction with 50 μ M IPTG, a 10% dodecane top layer was added directly after induction to create a two-phase extractive culture. At the point of analysis, it was observed that 35 mg/L of geraniol was present in the organic top layer (Figure 5.4). Interestingly, in addition to producing geraniol, this strain also produced significant amounts of the monoterpenoids nerol and citronellol, as well as the sesquiterpenoid farnesol, which were detected using GC-MS (Figure 5.4). Previous work has determined that the GES enzyme from *O. basilicum* has strict substrate specificity for synthesizing only geraniol from the geranyl pyrophosphate precursor (Iijima *et al.*, 2004), suggesting that the observation of additional terpene products is the result of modifications made to geraniol by endogenous *E. coli* enzymes. Fisher *et al.*, (2013) previously demonstrated that upon GES expression the composition of the resulting terpene profile is heavily dependent upon the heterologous host in which it is being expressed. Ultimately, this further endogenous manipulation of geraniol by host enzymes is

limiting to industrial level production, and several groups have worked towards minimizing this bioconversion (Zhou *et al.*, 2014). Also hindering industrial microbial production of geraniol is its high cytotoxicity, which is characteristic of monoterpenes. While the exact mechanisms of geraniol toxicity are poorly understood its moderate water solubility is most likely a contributing factor – endowing it with the capacity to migrate across aqueous environments and interact with and damage lipid membranes and intracellular components (Trombetta *et al.*, 2005; Brennan *et al.*, 2012; Shah *et al.*, 2013). Even when being produced in a biphasic extractive culture, the moderate water solubility of monoterpenes allows a proportion to remain in aqueous solution, exerting toxic effect. Therefore, it is proposed that a chemical modification that leads to a decrease in the water solubility of a monoterpene may result in a concomitant decrease in its microbial toxicity by further promoting its sequestration into the organic layer of a two-phase culture. A simple and effective chemical modification to decrease the water solubility of geraniol would be to esterify it, as this would occlude the free hydroxyl group of the monoterpene alcohol, decreasing its capacity for hydrogen bonding and consequential moderate aqueous solubility. In considering potential end-point esters into which to sequester geraniol, geranyl acetate was an ideal candidate. The primary reasons for this being that the requisite acyl substrate to produce geranyl acetate, acetyl-CoA, is readily available in the cell – thus substrate feeding or additional pathway manipulation/ heterologous expression would not be necessary. As well, there are a number of AAT enzymes that have been characterized with activity for producing geranyl acetate in the literature (D’auria *et al.*, 2002; Shalit *et al.*, 2003; Beekwilder *et al.*, 2004; Lucchetta *et al.*, 2007; Balbontin *et al.*, 2010).

First, to confirm that geranyl acetate would be extracted into the organic phase of a two-phase culture more efficiently than geraniol – the keystone to the success of this strategy – the relative organic and aqueous solubility of both compounds was assessed. This was done by incubating either geraniol or geranyl acetate for several hours in a biphasic culture under the same conditions used when culturing strain DLG2 (described above), followed by an analysis of the proportion of geraniol or geranyl acetate present in either the aqueous and organic phase. From this, it was observed that the esterified form of geraniol possessed significantly reduced water solubility compared to the non-esterified form. An analysis of the proportion of each compound found in the organic and aqueous phases of the two-phase system showed that 4% of geranyl acetate was present in the aqueous layer with the rest partitioning into the organic phase, whereas 34% of geraniol remained in the aqueous phase (Table 5.1). These results support the

use of geranyl acetate as the end-point monoterpene ester as it is partitions nearly entirely into the organic phase.

Table 5. 1 Percent geraniol and geranyl acetate present in the aqueous and organic layers of the two-phase system.

	% Aqueous	% Top Layer
Geraniol ^a	34 \pm 3	66 \pm 7
Geranyl acetate ^a	4 \pm 0.6	96 \pm 2

^a TB+2% glucose with a 10% dodecane top layer was supplemented with 5 mM geraniol or geranyl acetate and incubated for 3h before analysis. Data are the mean \pm standard deviation from three biological replicates.

In order to engineer *E. coli* to further convert endogenously made geraniol to geranyl acetate, the expression of an AAT enzyme is required. Of the AAT enzymes that have been characterized with activity for the synthesis of geranyl acetate, most possessed promiscuous substrate specificity, which is undesirable for the purposes of this work. One enzyme, however, from Rose (*R. hybrida*) had been shown to possess nearly exclusive activity for catalysing the esterification of acetyl-CoA to geraniol (Shalit *et al.*, 2003) (Figure 5.1), and thus was carried forward. The coding sequence of this AAT (*RhAAT*) was codon-optimized to the genome of *E. coli* and cloned into a high copy pET plasmid to drive expression under the strong T7 promoter (Methods Table 2.1; optimized sequence in Appendix). In order to confirm the previously documented specificity of this AAT enzyme for producing geranyl acetate, a single phase culture of *E. coli* strain C43 (DE3) heterologously expressing the pET28a::*RhAAT* plasmid was induced with 0.4 mM IPTG, exogenously fed 0.5 mM geraniol and 10 mM acetic acid two hours post induction, and cultured for 18 hours. Product analysis at 18 hours confirmed that geranyl acetate was nearly exclusively produced in these cultures (Figure 5.3), with only trace amounts of citronellyl-, neryl- and linallyl acetate detected by GC/MS (data not shown). Geranyl acetate was present in both the supernatant and pellet fractions, as no second organic extractive phase

was used, and the production of geranyl acetate had no significant impact on the OD₆₀₀ of cultures compared to wild type *E. coli* C43 (DE3) producing no monoterpene ester. Ultimately, this suggests that *RhAAT* is an ideal alcohol acyltransferase for the purposes of this work as it displays high product specificity.

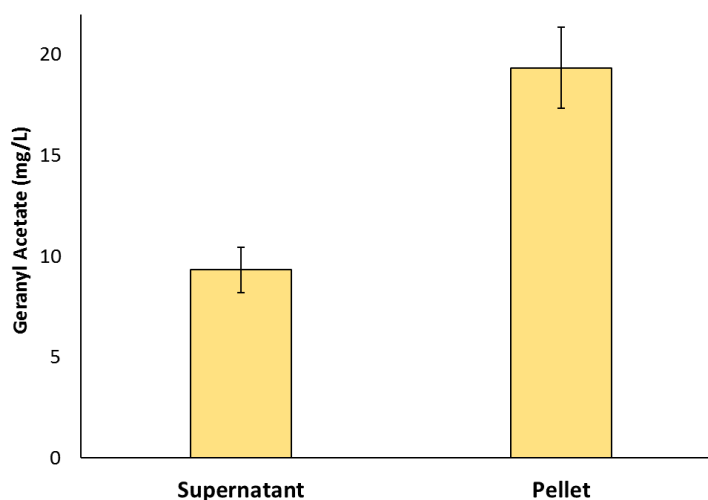


Figure 5. 3 Geranyl acetate accumulated in the supernatant and cell pellet of *E. coli* cultures expressing an AAT from *R. hybrida*. Cultures were exogenously fed acetic acid and geraniol after induction and grown in a solvent-free system for 18 hours at 20°C before analysis. Data are the mean \pm standard deviation from three biological replicates.

With the successful construction of a plasmid that possesses the requisite genes for geraniol production (pMIB13), and a second plasmid that harbours an AAT enzyme with high specificity for acetylating geraniol (pET28a:*RhAAT*), *E. coli* C43 (DE3) was co-transformed with both plasmids to generate the geranyl acetate producing strain, DLGA3 (Methods Table 2.1). Interestingly, expression of strain DLGA3 produced high titres of exclusively geranyl acetate (350 mg/L) from glucose after 24 hours of fermentation, post induction with 0.1 mM IPTG, in a two-phase culture (Figure 5.4). A second organic phase was used in geranyl acetate producing cultures as it was found that high concentrations of geranyl acetate supplemented into cultures of

wild type *E. coli* C43 (DE3) impacted growth compared to a no geranyl acetate addition control over the course of incubation (Appendix, Supplementary Figure 9). A GC-MS analysis of the culture supernatants after 9 hours of incubation with 10 mM geranyl acetate showed the presence of geraniol in addition to the acetate ester (data not shown), suggesting that promiscuous endogenous esterases were hydrolysing geranyl acetate to produce the highly toxic geraniol. Geranyl acetate was quantified exclusively from the organic phase, and cultures had no additional substrate supplementation with either geraniol or acetic acid. As well, no geraniol was detected in these cultures, suggesting that all produced geraniol had been converted to geranyl acetate. Overall, strain DLGA3 shows a marked improvement in product formation over strain DLG2 which was previously shown to produce a maximum of 35 mg/L of geraniol from glucose after 24 hours – as well as a number of other terpene end-products (Figure 5.4). A comparison of the geraniol titres achieved by the two strains showed that DLGA3 produced approximately 8-fold more geraniol than DLG2. A partial explanation for the difference in product titre between strains DLG2 and DLGA3 can be related back to the respective organic phase solubilities of geraniol and geranyl acetate. As only those products present in the organic top layer were

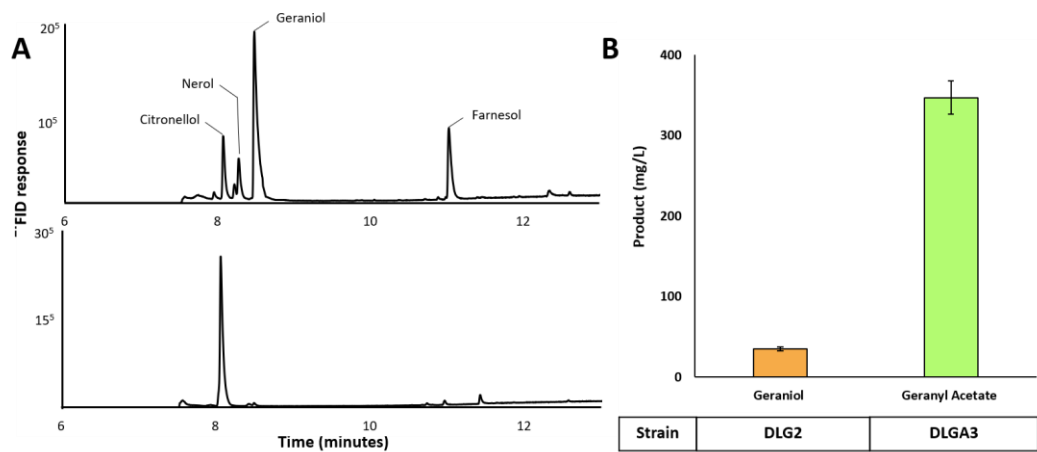


Figure 5. 4 A. Gas chromatographs of the dodecane phase of two-phase cultures of *E. coli* strains DLG2 (top panel) producing several terpenols, and DLGA3 (bottom panel) producing only geranyl acetate. **B.** The total production (mg/L) of either geraniol or geranyl acetate produced by strain DLG2 and DLGA3, respectively. Cultures were induced with IPTG, followed by the addition of a 10% dodecane top layer for product extraction. Product analysis occurred after 24h of growth at 30°C. Chromatograph peaks for citronellol, nerol, geraniol, farnesol, and geranyl acetate are indicated. Data are the mean \pm standard deviation from three biological replicates.

quantified, the proportion of the geraniol produced by strain DLG2 that remained in the aqueous phase had not been factored in. However, this end-product solubility alone is not a complete explanation for the significantly higher titre achieved by strain DLGA3. Even extrapolating for the total amount of geraniol produced by DLG2 – taking into consideration the amount not extracted into the top layer using the aqueous phase solubility found in table 5.1 as a guide – resulted in a total of 47 mg/L of geraniol, approximately 6-times less than the amount of geraniol produced by strain DLGA3. A potential alternative explanation for the disparity in product formation between the two strains relates to intrinsic pathway regulation. It may be the case that geraniol acts as an end-product feedback inhibitor to the mevalonate pathway, and that its accumulation in the aqueous phase of cultures of strain DLG2 allows it to exert this activity, limiting further geraniol production. Whereas in strain DLGA3, where geraniol is further sequestered into an acetate ester that is efficiently extracted into the organic layer, end-product feedback inhibition is alleviated, therefore driving further production. Should geraniol be involved in negative feedback inhibition of the MEV pathway, the most likely protein it would interact with would be the terminal GES enzyme. The reason for this being that all upstream enzymes in the MEV pathway are communally used for the production of all terpenes, many of which are integral to cell form and function, and thus unlikely to have their expression regulated by a single end product. To assess the potential inhibition of geraniol on GES enzyme activity, the optimized GES gene was amplified without a stop codon cloned into the MCS of an empty pET28a plasmid upstream of a His6x tag. The GES enzyme was subsequently purified using cobalt resin from cultures expressing the pET21a::GES, and used for *in vitro* enzyme assays assessing GES activity for synthesizing geraniol in the presence of increasing concentrations of geraniol with the colorimetric Malachite Green assay. It was observed, however, that even at the highest geraniol concentrations tested, there was no significant impact on GES activity (Table 5.2), as is evident by the unchanged V_{\max} , K_m , and k_{cat}/K_m values observed for the GES activity while in the presence of increasing concentrations of geraniol as a potential inhibitor. Should there be any product inhibition, depending on the type, these values would be changed from the 0 μM geraniol control. This suggests that the presence of geraniol is not inhibitory towards the activity of this enzyme. However, though unlikely, this does not exclude the possibility that geraniol may interact with and inhibit one of the enzymes upstream in the MEV pathway, as this pathway has been well documented in the literature to be subject to complex regulation (Dimster-Denk *et al.*, 1999; Primak *et al.*, 2011).

Table 5. 2 *In vitro* activity of the GES enzyme from *O. basilicum* converting GPP to geraniol in the presence of varying concentrations of geraniol. Kinetic parameters were determined using the malachite green assay in the presence of increasing geraniol concentrations. Data are the mean \pm standard error from three replicates.

	Geraniol (μM)			
	0	100	200	400
V_{\max} ($\mu\text{mol/min/mg}$)	1.7 (\pm 0.2)	1.6 (\pm 0.2)	1.5 (\pm 0.1)	1.6 (\pm 0.1)
K_m (μM)	121 (\pm 16)	96 (\pm 10)	98 (\pm 12)	98 (\pm 14)
k_{cat} (s^{-1})	1.8 (\pm 0.2)	1.7 (\pm 0.2)	1.6 (\pm 0.1)	1.7 (\pm 0.1)
k_{cat}/K_m ($\mu\text{M}^{-1}/\text{s}^{-1}$)	0.016	0.018	0.017	0.018

In addition to the improved product titres observed in strain DLGA3, there was also a marked improvement in product specificity – with geranyl acetate being the sole end-product (Figure 5.4). This, again, is most likely a result of the reduced water solubility of geranyl acetate compared to geraniol. In strain DLGA3, the geranyl acetate produced is extracted more efficiently into the organic phase and therefore less available for manipulation by endogenous *E. coli* enzymes, unlike geraniol which accumulates in the aqueous phase to a larger proportion and is subsequently converted to other monoterpenoids (Figures 5.4 & 5.8). Ultimately, what is observed is that the esterification of geraniol is beneficial not only for improved product formation, but also for improved product specificity, the two primary problems hindering geraniol production on an industrial scale.

5.2.2 Acetic acid feeding to improve Geranyl Acetate production

To further improve geranyl acetate titres we considered pathway bottlenecks. While the MEV pathway used here had been previously optimized to balance gene expression and flux of intermediates (Alonso-Gutierrez *et al.*, 2013), accommodations for the increased consumption of

acetyl-CoA had not been taken into consideration. Acetyl-CoA is a node from which much cellular metabolism branches and the introduction of a heterologous pathway that requires seven acetyl-CoA molecules for each geranyl acetate molecule imposes a significant demand on flux through this node. Previously it has been shown that genetic manipulations aimed at increasing acetyl-CoA precursor availability in *E. coli* and *S. cerevisiae* have resulted in improved product titres of the terpenes lycopene and amorphadiene, respectively (Alper *et al.*, 2005; Shiba *et al.*, 2007). As an alternative approach, here we investigated the viability of media supplementation with acetic acid as a means to increase the abundance of available acetyl-CoA. When grown in the presence of fermentable sugars, *E. coli* converts acetyl-CoA to acetate through the action of the reversible PTA-ACK pathway (using phosphotransacetylase/acetate kinase), with the acetate produced being subsequently excreted (Wolfe, 2005). It has been found, however, that the direction of the PTA-ACK pathway is dependent upon the extracellular concentration of acetate, with acetate being assimilated by *E. coli* when present in an external concentration above a threshold value of 8 mM, even in the presence of excess glucose (Enjalbert *et al.*, 2017). This suggests that the exogenous addition of acetic acid in *E. coli* cultures grown on glucose may be used as a strategy to increase the pool of intracellular acetyl-CoA as the driving force of the PTA-ACK pathway is pushed towards its synthesis, resulting in acetyl-CoA accumulation both from the consumption of glucose and acetate simultaneously (Castaño-Cerezo *et al.*, 2009; Krivoruchko *et al.*, 2015; Yang *et al.*, 2016; Enjalbert *et al.*, 2017). Strain DLGA1 was cultured in media supplemented with either no acetic acid, 5 mM, 10 mM, or 20 mM acetic acid, which was added immediately post induction. After 24 h, cultures fed no acetic acid had produced 585 mg/L of geranyl acetate while the cultures supplemented with 20 mM acetic acid produced 940 mg/L, a 60% increase in product titre (Fig. 3). Those fed 5 mM and 10 mM produced intermediate amounts of geranyl acetate (Fig. 3). This clearly demonstrates that the production of geranyl acetate in these strains is limited by the availability of acetyl-CoA, either as a precursor for geraniol biosynthesis or for acetylation of geraniol, and that further increase in flux to geranyl acetate by increasing acetyl-CoA production should be possible. Although high concentrations of acetic acid are detrimental to cell growth as it can uncouple the transmembrane pH gradient and inhibit methionine biosynthesis (Roe *et al.*, 2002; Wolfe, 2005), media supplementation with up to 20 mM acetic acid (1.2 g/L) had no significant impact on final culture OD₆₀₀, probably due to the buffering capacity of TB media. (Fig. 3).

Further improvement on this acetic acid supplementation strategy may be achieved by de-regulating expression of an acetyl-CoA synthase (ACS) enzyme – which catalyses the direct

condensation of acetate and coenzyme A (CoA). Transcription of the endogenous *E. coli acs* is repressed in the presence of glucose (Krivoruchko *et al.*, 2015). Yang *et al.*, (2016) have recently shown that dual expression of an ACS from *Acetobacter pasteurianus* and an acetoacetyl CoA synthase (AACS) from *Streptomyces sp. strain CL190* in an *E. coli* strain engineered for β -caryophyllene production results in improved product titres when acetic acid is used as the sole carbon source.

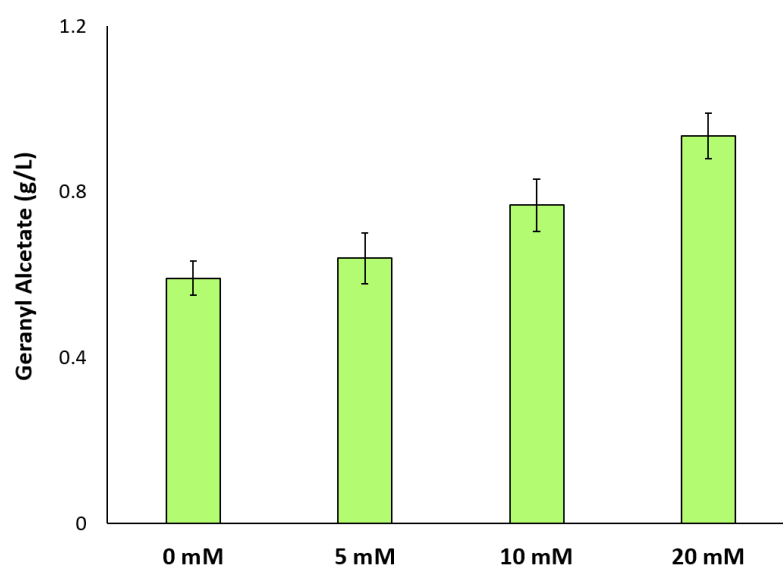


Figure 5. 5 Geranyl acetate production by *E. coli* strain DLGA2 after 24h when supplemented with 0 mM, 5 mM, 10 mM or 20 mM acetic acid. Following induction with 100 μ M IPTG, cultures were overlaid with a 10% dodecane top layer, and fed +/- acetic acid before being incubated at 30°C. Average culture OD₆₀₀ for no acetic acid feeding and 20 mM acetic acid feeding are 33 and 36, respectively. Data are the mean \pm standard deviation from four biological replicates.

5.2.3 Geraniol and geranyl acetate formation under fed-batch conditions

To scale up geraniol and geranyl acetate production, fed batch fermentations of strains DLG2 and DLGA3 were performed in a 1.5 L batch reactor. Both strains were cultured in a modified

TB media (MTB) in which the phosphate buffer salts were replaced with sodium chloride; this substitution was made as the culture pH would be maintained at 6.8 by the addition of acid and base. Cultures were grown at 30°C and induced with either 50 μ M IPTG (strain DLGA2) or 125 μ M IPTG (strain DLGA3) when culture OD₆₀₀ reached 20. Culture OD₆₀₀ and product formation were tracked over the course of 115 hours for both strains. With strain DLG2, geraniol production peaked at 34 hours with 220 mg/L, after this culture geraniol decreased while citronellol and nerol increased (Figure 5.6A). An analysis of total monoterpenoids suggests that after the peak in geraniol at 34 hours, the total amount of terpene remains relatively stable, with only the proportion of each monoterpenoid altering over time. This would suggest that the formation of citronellol and nerol are not *de novo*, but rather a result of the biotransformation of geraniol by endogenous *E. coli* enzymes. Figure 5.7 shows the proposed scheme for the bioconversion of geraniol to nerol and citronellol. To support this model, wild-type C43 (DE3) *E. coli* was incubated in the presence of either 0.5 mM geraniol or nerol for 6 hours and then evaluated the terpene profiles of each culture by GC-MS (Figure 5.8). It was observed that despite the incubation of these cultures in the discrete presence of one monoterpene, the presence of all three – geraniol, nerol, and citronellol – was detected, supporting the proposed scheme. When wild-type C43 (DE3) *E. coli* was incubated in the presence of 0.5 mM citronellol no biotransformation to other terpenes was observed (data not shown); an occurrence that has been previously reported in *S. cerevisiae* as well (King and Dickinson, 2000). Previously, several groups have identified possible enzymes responsible for the reduction, isomerization and dehydrogenation of geraniol in *E. coli*, *S. cerevisiae*, and other organisms (Steyer *et al.*, 2013; Zhou *et al.*, 2014; Liu *et al.*, 2016). Interestingly, over the course of the batch fermentation of strain DLG2, citronellol became the dominant terpene product, culminating in 290 mg/L at 112 hours with minimal amounts of geraniol and nerol present at this time point (Figure 5.6A). No enzyme has been identified that is capable of converting citronellol to either geraniol or nerol. From data, it may be possible that GES expression in *E. coli* could be used not only as a means for geraniol production, but that it may also be considered as a platform for citronellol production.

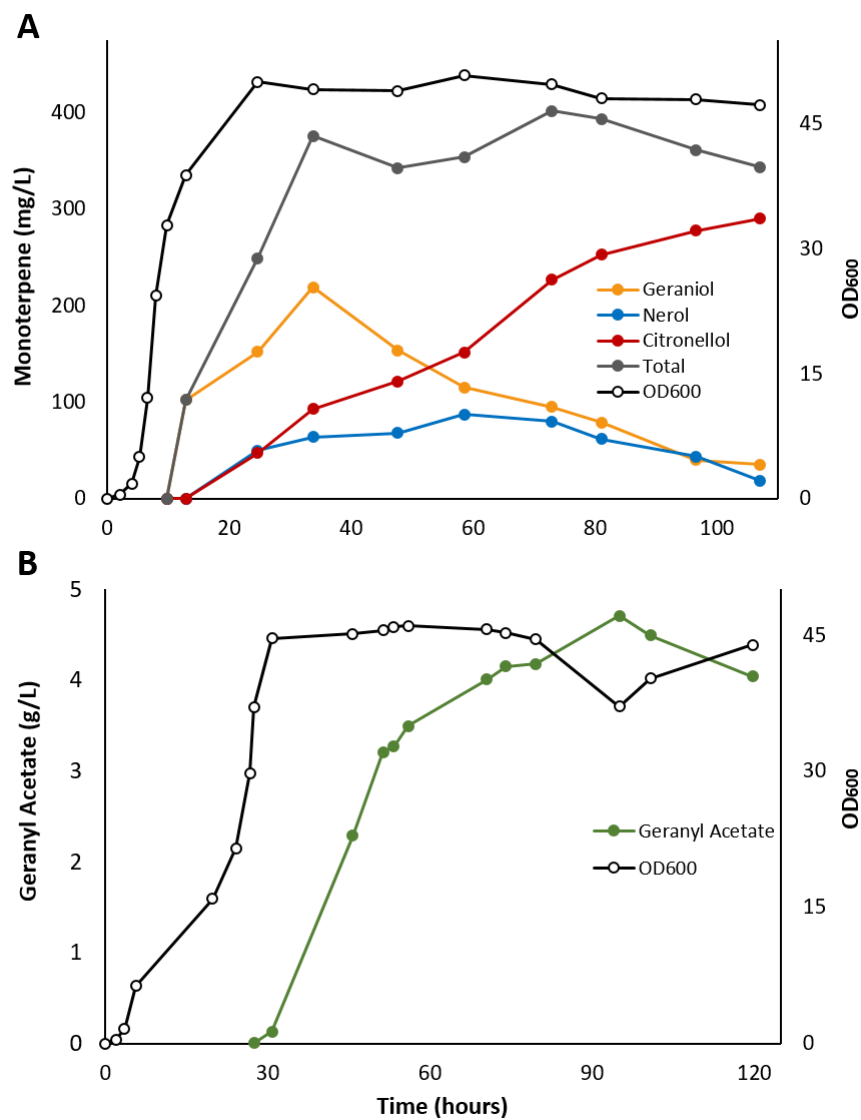


Figure 5. 6 Monoterpene and monoterpene ester production in *E. coli* under fed-batch fermentation using a two-phase system. **A** Accumulation of geraniol (purple), nerol (blue), citronellol (red) and total monoterpene (grey) in *E. coli* strain DLG2. **B** Accumulation of geranyl acetate (green) in *E. coli* strain DLGA3. Both cultures were induced at an OD₆₀₀ of ~20 with either 50 μ M IPTG (A) or 125 μ M IPTG (B), followed by the addition of a 10% dodecane top layer. Culture (B) was further supplemented with 20 mM acetic acid.

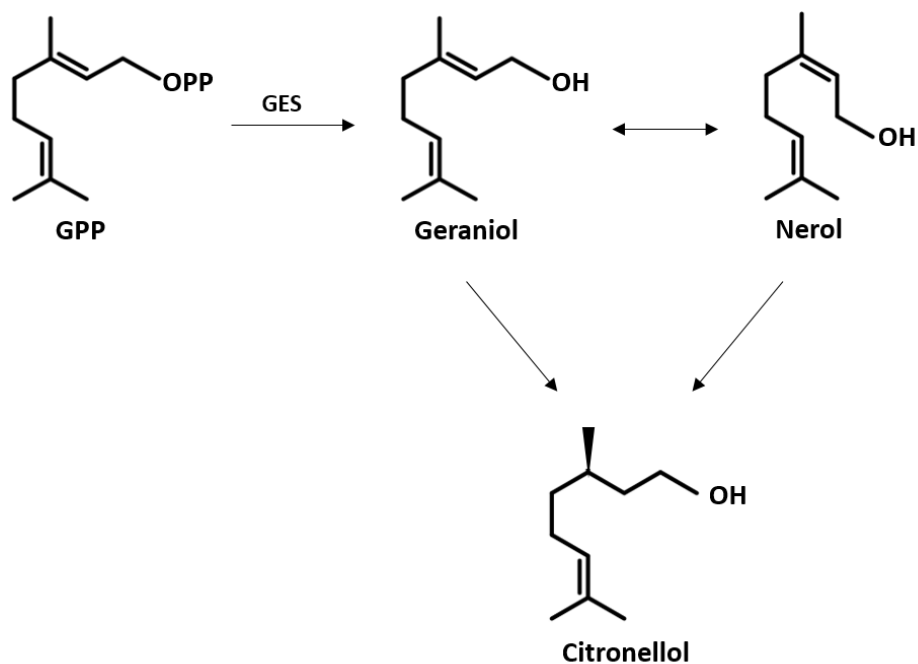


Figure 5. 7 Proposed pathway for the conversion of geraniol to the similar monoterpenoids, nerol and citronellol, by endogenous *E. coli* enzymes. The GES enzyme from *O. basilicum* has exclusive substrate specificity for converting GPP to geraniol. In *E. coli* culture expressing the GES enzyme, the observed accumulation of both nerol and citronellol over time is most likely the result of endogenous enzymes catalysing the isomerization of geraniol to nerol and vice versa, as well as the reduction of both species to citronellol.

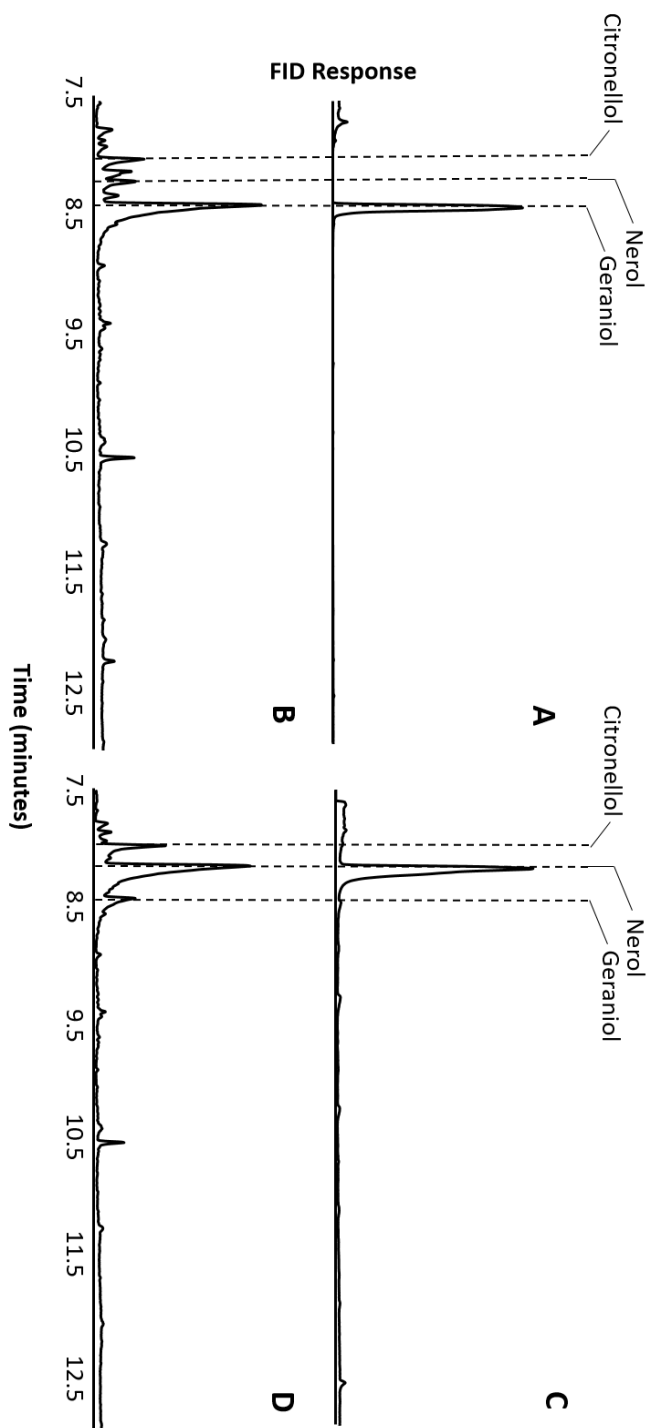


Figure 5. 8 Gas chromatographs showing the monoterpene profile of *E. coli* C43 (DE3) after incubation in the presence of either geraniol or nerol. Panels **A** and **C** show internal standards of geraniol and nerol, respectively. Panel **B** and **D** show the monoterpene profile of wild type *E. coli* C43 (DE3) incubated in the presence of either 0.5 mM geraniol or 0.5 mM nerol respectively, for 6 h. The peaks corresponding to citronellol, nerol and geraniol are indicated.

As previously observed in shake flask fermentation, strain DLGA3 produced exclusively geranyl acetate in a fed-batch bioreactor run with MTB media. The culture was fed 20 mM acetic acid directly post induction, as it was previously shown to increase product titre (Figure 4). Geranyl acetate titres peaked at 95 hours with 4.8 g/L (approximately 100 mg/OD unit) (Figure 5.6B). This is approximately 2.4 times more geranyl acetate than the previously reported highest titres achieved by Liu *et al.*, (2016), where bioconversion of geraniol to geranyl acetate, via the unexpected activity of a promiscuous chloramphenicol acetyltransferase (CAT) enzyme, was also used. Further, strain DLGA3 was also cultured in a fed-batch bioreactor using a semi-defined media (FM) that is more typical of industrial production. Here, geranyl acetate production peaked at 77 hours with 422 mg/L (approximately 50 mg/OD unit) (Figure 5.9), and again was the only product observed over the course of the fermentation. Though these titres are lower than those observed in the rich MTB media (Figure 5.6B), further optimization of culturing conditions will improve strain production.

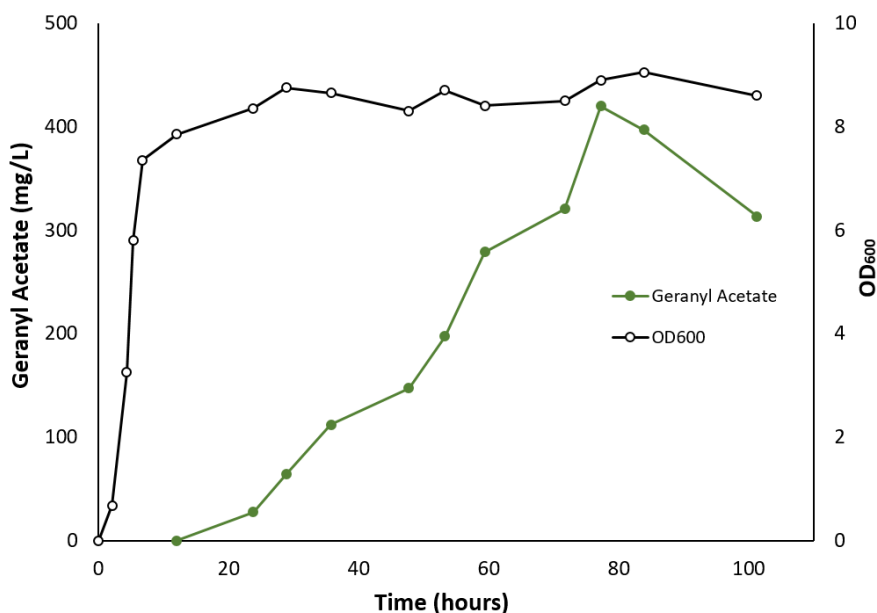


Figure 5. 9 Production of geranyl acetate in *E. coli* strain DLGA3 under fed-batch fermentation using a two-phase system with a semi-defined media. The culture was grown in a fermentation media +2% glucose at 30°C and induced when OD600 reached ~5.5 using 100µM IPTG, followed by the addition of a 10% dodecane top layer and media supplementation with 20 mM acetic acid.

5.3 Conclusion

Here, a rationally developed *E. coli* platform for the detoxification of the monoterpene geraniol through its esterification into an acetate ester. Using this strategy, 4.8 g/L of geranyl acetate can be produced from glucose in fed batch culture. This represents nearly a 2.5 times improvement from previous geraniol producing systems, which had been hindered by the high microbial toxicity of geraniol (Shah *et al.*, 2013; Zhou *et al.*, 2014). As well, here it has been shown that sequestering geraniol into an acetate ester results not only in higher product titres, but also eliminates the bioconversion of geraniol to other monoterpenoids, which has been a continuing obstacle to industrial production. Ultimately, this work provides a strategy for the improved production of other commercially valuable monoterpenoids, such as terpineol, linalool, fenchol, and perillyl alcohol (POH) using this ‘detoxification via esterification’ approach. Further metabolic engineering efforts to improve acetyl-CoA and cofactor availability, and the characterization of additional AAT enzymes will aid in developing this strategy as an economically feasible microbial production platform for monoterpenoids.

6. General discussion and conclusions

The development of microbes as industrial platforms for the synthesis of commercially valuable compounds requires high titre product formation for the process to be economically viable. The high titres required, however, often exceed the native tolerance levels of the microbial host, creating an undesirable trade-off between culture health and productivity that ultimately limits the attainable yields (Dunlop *et al.*, 2011). As such, a large amount of research has gone into developing stress-tolerant strains and *in situ* end-product removal strategies to minimize the toxic impact that the accumulation of the desired end-product exerts on the microbial host; with the ultimate goal of improving achievable titres in culture and creating more cost-effective platforms. This project aimed to investigate an alternative strategy for the detoxification of value compounds in the bacterial platform host, *E. coli*, that involves *in vivo* sequestration. Unlike the sequestration strategies currently used in industrial fermentations, which aim to continuously remove the end-product from the culture to minimize its accumulation and exposure to the cells, this strategy focused on engineering the microbial host to further incorporate the toxic end-product into a more neutral compound that exerts little to no toxicity on the host, even at high concentrations. The category of toxic end-product used as a model in this work was an alcohol, while the more neutral molecule into which it was incorporated was an ester. Alcohol esterification was the chosen strategy for detoxification in this work as it is a simple single step reaction that requires only the activity of a single enzyme to catalyse the condensation of an alcohol to an acid. As well, there is no significant change in structure to the alcohol component, but rather it can easily be recovered downstream from the ester molecule through hydrolysis. To investigate the effectiveness of this strategy for the detoxification of commercially valuable alcohols in *E. coli*, it was applied to the production of two toxic alcohols: butanol and geraniol. For geraniol, this strategy proved to be highly effective, whereas for butanol it showed minimal success. A comparison of several factors illustrates the reasons for the discrepancy in the effectiveness of this strategy towards these two alcohols.

The first factor influencing the success of this strategy involved the substrate specificity of the AAT enzyme responsible for the esterification reaction. In chapter 3, it was identified that the

shortest chain aliphatic butyl ester to exert no toxicity at high concentrations in *E. coli* cultures, was butyl octanoate. All butyl esters of shorter acyl chain examined negatively impacted the growth rate of *E. coli* at lower concentrations than butanol – thus butyl octanoate became the target end-point ester for this detoxification strategy. Identifying an AAT enzyme capable of esterifying butanol and octanoic acid became difficult as most of the AAT enzymes that have been characterised possess substrate specificity for shorter acyl chain substrates (C2:0 and C4:0) (Table 4.1), and for those AATs that do possess activity towards longer acyl chains, they are often not the preferred substrate. This was the case for the *EcAAT* enzyme from *A. chinensis* that was used for butyl octanoate production in this work. Exogenous feeding of butanol and octanoic acid into cultures of *E. coli* expressing the *EcAAT* resulted in a small amount of butyl octanoate, but significantly more butyl butyrate, and butyl hexanoate – as this *EcAAT* from *A. chinensis* prefers the shorter butyrate and hexanoate substrates over octanoate (Günther *et al.*, 2011). This is especially problematic as both of these shorter chain butyl esters are toxic to *E. coli* at low concentrations. The two potential solutions to this problem of product specificity would be either: (i) significant engineering the *EcAAT* enzyme to alter its substrate specificity to exclusively accept the octanoate chain length (this could be paired with metabolic engineering to increase octanoyl-CoA abundance intracellularly), (ii) or to identify a different AAT enzyme with stricter substrate specificity for producing butyl octanoate, and not the shorter chain esters. While the latter option would be the ideal solution, to date no AAT enzyme with this specific substrate specificity has been characterised. Efforts to engineer the *EcAAT* enzyme to improve its specificity for the octanoate chain length in chapter 4 were minimally successful; while two individual point mutations did result in a small increase in butyl octanoate production, neither mutation resulted in a decrease in toxic butyl hexanoate or -butyrate production – negating the effectiveness of the esterification strategy for detoxifying butanol. Future efforts aimed at engineering AAT enzymes would benefit significantly from a crystal structure, as currently none is available. Unlike the *EcAAT* enzyme, however, the *RhAAT* from *R. hybrida* displayed high specificity for producing geranyl acetate. While Shalit *et al.*, (2013) demonstrated *in vitro* that the *RhAAT* enzyme has low activity towards acetylating citronellol and nerol alcohols as well, expression of *RhAAT* in geraniol producing *E. coli* cultures esterified geraniol to geranyl acetate more quickly than endogenous *E. coli* enzymes could convert geraniol to either citronellol or nerol – thus preventing the accumulation of these substrates for the *RhAAT* to acetylate. Thus, the high geraniol substrate specificity of the *RhAAT* enzyme contributed to the improved product titres and product specificity achieved for *E. coli* in Chapter 5. Ultimately, the

successful application of esterification as a strategy for detoxification relies on the use of an AAT enzyme with high specificity for producing the desired end-product.

The second factor influencing the successful application of this strategy is the chain length of the acid substrate required for esterification of the alcohol into a nontoxic ester. The octanoyl-CoA substrate needed to produce butyl octanoate has low natural abundance in *E. coli* as it is typically an intermediate product in the β -oxidation of long chain fatty acids for acetyl-CoA production, or a substrate for incorporation into other lipid compounds (Iram and Cronan, 2006; Torella *et al.*, 2013). This was problematic as the acyl-CoA substrate for this esterification strategy should not be limiting. Thus, to improve intracellular octanoyl-CoA availability in *E. coli* for butyl octanoate formation via *EcAAT* activity, either exogenous culture supplementation with octanoic acid, or engineering of *E. coli* lipid metabolism for increased intracellular octanoyl-CoA, is required. Both of these strategies were explored in Chapter 4. Exogenous supplementation of *E. coli* cultures expressing the *EcAAT* with 5 mM octanoic acid was effective for butyl ester formation and did not negatively impact on culture health. However, product analysis after 18 hours of incubation showed that all exogenously supplemented octanoic acid had been consumed, suggesting it may be a limiting substrate for higher butyl ester production. Culture supplementation with higher concentrations of octanoic acid, however would be detrimental as Royce *et al.*, (2013) showed that the addition of 10 mM octanoic acid has a significant impact on *E. coli* culture density. Thus, for exogenous octanoic acid supplementation to be effective, a balance between maximizing product formation and minimizing culture toxicity must be established. To achieve this, octanoic acid supplementation would need to be done gradually at a low concentration over the course of the fermentation, thus facilitating a constant surplus of octanoyl-CoA for the *EcAAT* enzyme, but not exceeding native *E. coli* tolerance to this acid. For this to be possible, careful monitoring of octanoic acid and butyl ester titres in culture – as well as culture pH – over the course of the fermentation would be required; a task that is difficult to do in shake flask. More difficult would be the engineering of *E. coli* metabolism to maximize endogenous octanoyl-CoA abundance, as this involves manipulation of the highly regulated processes of fatty acid synthesis and β -oxidation. The work done towards this end in Chapter 4 was unfinished, but the could prove to be effective in a $\Delta FadE$ knockout *E. coli* strain that possesses the phage T7 RNA polymerase gene. Whether such a strain could successfully produce high titres of butyl octanoate and minimal titres of butyl

hexanoate and -butyrate, however, is unlikely without improvement of the AAT enzyme activity and substrate specificity. Conversely, the acyl substrate required for geranyl acetate production via *RhAAT* activity in *E. coli* is acetyl-CoA; a substrate that is abundant in the cell as it is an essential intermediate in a number of biosynthetic pathway (Chohnan *et al.*, 1998). Geraniol producing *E. coli* cultures expressing *RhAAT* were capable of producing high titres of geranyl acetate from endogenous acetyl-CoA pools (i.e. no acetic acid supplementation). As well, these cultures did not appear to have reduced health; impressive as seven acetyl-CoAs are required to produce one geranyl acetate via the heterologously expressed MEV pathway and *RhAAT* enzyme – a significant investment of acetyl-CoA from the cell. The ability of *RhAAT* expressing *E. coli* cultures to produce geranyl acetate without the need for exogenous acyl substrate supplementation is a stark contrast to the cultures expressing the *EcAAT*, which produce no butyl octanoate without exogenous octanoic acid addition. Further, supplementation of *RhAAT* expressing cultures with 20 mM acetic acid improved geranyl acetate production by 60% over cultures that had no supplementation, and resulted in no observable impact on culture health. This is again in contrast to octanoic acid supplementation in *EcAAT* expressing cultures, which would become inhibitive at 10 mM. It would be interesting to see what the top limit of acetic acid supplementation is before becoming detrimental to culture health. Ultimately, the intracellular availability of acetyl-CoA, and tolerance for exogenous acetic acid supplementation, contributed to the observed high titre production of geranyl acetate in *E. coli*.

Third factor influencing the successful application of this detoxification strategy was the use of a second organic extractive phase. Cultures producing geranyl acetate were grown in the presence of a 10% dodecane layer for *in situ* extraction. This was done as it is common practise to culture terpene producing cultures with a second phase due to their high toxicity (Brennan *et al.*, 2012). This second organic phase was effective at sequestering approximately 96% of geranyl acetate produced, minimizing its exposure to the culture. This was beneficial for two reasons, first, it prevented the geranyl acetate from accumulating intracellularly. It was found in Chapter 5 that *E. coli* cultures producing geranyl acetate without the use of an organic extractive phase resulted in the majority of the product accumulating in the cell pellet fraction after centrifugation. Second, it minimized the exposure of geranyl acetate to endogenous *E. coli* enzymes. Such exposure could result in undesirable manipulations of the geranyl acetate, such as hydrolysis by non-specific esterases, or biotransformations of the geraniol component, as was seen in the

geraniol producing cultures. For butyl ester production in the *EcAAT* expressing *E. coli* cultures, however, a second organic phase was not used. The reason for this being, that unlike in the geraniol producing cultures, the titres of butanol and butyl ester being achieved did not exceed *E. coli* tolerance limits. The use of an organic top-layer for butyl ester sequestration in *EcAAT* expressing cultures would be possible should an organic solvent be identified into which the ester products readily partition, while the butanol and acid substrates do not. Layton *et al.*, (2016) demonstrated the effective use of hexadecane as an organic phase in *E. coli* cultures producing short chain esters. The use of a top layer in this work would be additionally beneficial as it would mean that engineering of the *EcAAT* enzyme to decrease its activity for producing the shorter chain butyl butyrate and -hexanoate esters would no longer be required. Instead, these toxic end-products would be extracted into the organic phase, minimizing their exposure to the culture. However, the original goal of this work was to develop a stand-alone detoxification strategy for butanol that would require no additional detoxification techniques, such as the use of an extractive phase that would increase industrial fermentation costs. This is in contrast to the work done by Layton *et al.*, (2016), for whom ester production was the end goal.

The question remains, however, whether this esterification strategy would be effective for butanol detoxification. The answer is most likely, no. Currently the highest titre production of butanol in *E. coli* flask cultures is 15 g/L after 75 hours (Shen *et al.*, 2011), while the highest titre of total butyl ester achieved in this work – including all three butyl esters chain lengths present in both the pellet and supernatant fractions – was 165 mg/L after 18 hours. Not only that, but this is the butyl ester titre achieved in cultures that were exogenously fed both the butanol and octanoic acid substrates, as the titres achieved by cultures endogenously producing butanol were significantly lower. For this esterification strategy to be effective for butanol detoxification, it would require that more than 15 g/L of ester be achieved after 75 hours of culturing, which is 90 times more ester than what was produced by *E. coli* after 18 hours in this work. Achieving this is unlikely as it would require an immense amount of strain engineering as well as a resolution of the AAT/Ter co-expression problem. Instead, the development of a cost-effective microbial platform for butanol production that is able to compete with petroleum derived fuels and chemicals will most likely be achieved by optimizing multiple aspects of butanol fermentation, and not just toxicity alone. Gas stripping as a form of *in situ* extraction has been very effective for butanol recovery in both *E. coli* and clostridial fermentation cultures

(Shen *et al.*, 2011; Xue *et al.*, 2012), and optimization of factors such as gas recycle rate and bubble size could further improve gas stripping as a technique for butanol recovery (Zheng *et al.*, 2009). Further, the high butanol titres achieved in microbial fermentations have used glucose as the carbon source, an expensive substrate. The use of low cost substrates such as lignocellulose would be ideal for butanol production, and engineering strains for more efficient bioconversion of cellulose and hemicellulose would increase the economic success of industrial production of butanol (Zheng *et al.*, 2009; Chen *et al.*, 2016). It will be interesting to see in the future whether Clostridia or *E. coli* is favoured as an industrial butanol producing host. While clostridial strains such as *C. acetobutylicum* are currently able to produce and tolerate more butanol than the heterologous host *E. coli*, the genetic tools available to clostridia are less advanced and it more difficult to culture.

While esterification of butanol may not be the next big breakthrough in tolerance engineering, medium chain butyl esters such as those produced in this work have their own commercial value as flavour and fragrance additives. While several groups have engineered *E. coli* for the synthesis of short chain ester (Rodriguez *et al.*, 2013; Guo *et al.*, 2014; Tai *et al.*, 2015), very little work has gone into developing strains for the production of medium chain butyl esters, and those that have been produced in *E. coli* have been low in titre (Layton *et al.*, 2016). As was explored in the appendix chapter with clostridia, the production of butyl esters in *E. coli* could be used as a platform for these valuable flavour and fragrance additives, instead of as a butanol detoxification strategy.

While the use of esterification for the detoxification of butanol in *E. coli* was not successful, the application of this strategy to the detoxification of the monoterpene geraniol was highly effective, and resulted in an increase in both product titre and specificity. Previous attempts to alleviate monoterpene toxicity have had minimal success (Shah *et al.*, 2013), and this work represents the highest titres achieved for geraniol to date. The application of this strategy to the production of other monoterpene alcohols in *E. coli* would likely also be successful in improving titres. Potential future candidates for this would be citronellol, linalool, terpineol, lavandulol, and perillyl alcohol; each of which has commercial value (Rottava *et al.*, 2011; Alonso-Gutierrez *et al.*, 2013; Amiri *et al.*, 2016). The bottleneck, however, is the availability of AAT enzymes that have been characterised as high activity for esterifying these monoterpene alcohols. While several have been shown to work accept perillyl alcohol and linalool (D'auria *et*

al., 2002; Beekwilder *et al.*, 2004; Shalit *et al.*, 2013), they are not preferred substrates. Further fundamental work needs to be done to identify and characterise novel AAT enzymes from plants that produce these monoterpene products. For instance, the plant *Lavandula x intermedia* produces both lavandulol and lavandulyl acetate, however the AAT enzyme responsible for this esterification reaction has not yet been identified (Sarker *et al.*, 2015).

Ultimately, the use of esterification for the detoxification of value alcohols – or, just as equally, acids – is not a universal solution for microbial toxicity. Its success is dependent upon a number of factors, such as the availability of substrate and the specificity of the AAT enzyme.

7. References

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8. Appendix

Codon harmonized DNA sequence of *A. chinensis* AAT to the genome of *E. coli*

ATGGCGTCTTTTCCGCCTTCGTTAGTTTTACAGTTCGCCGCAACGAGCCTACGCTGGTGTTA
CCTAGCAAATCGACGCCGCGGAATTAAAACAGTTATCTGACATAGATGATCAGGAAGGTTT
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CTGTAAAGGTAATCCGCGAAGCGTTAGCGGAAGCGCTGGTGTTTTACTACCCGTTTCGCAGGT
CGCCTGATTGAGGGTCCAAATCGGAAGCTGATGGTGGATTGTACAAGTGAAGGGGTGTTATT
CATAGAGGCGGATGCGGATATTGAGGTCAATCAACTCATTGGTGACACGATTGATCCGGGTT
TTAGCTACCTCGACGAGCTGCTGCATGACGTGCCGGGGAGCGAAGGTATCCTGGGGTGTCCG
TTACTGCTGATTCAAGTTACGCGCTTTTCGCTGTGGTGGTTGGGCTTTTGCAATACGACTCAAT
CACACGATGTCTGATGCCCCGGGGTTAGTGCAGCTTCTTACCACCATTGCGGAGTTTGC GCG
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AGCCGCCGTCGATCACGTTCCAACATCACGAATACGAGCAGGTTATCAATACGACGACAGAT
GACAATAAGAGTATGACGCACAAAAGCTTTTTTTTCGGTCCAAAAGAGATACGCGCAATTTCG
GAGTCATTTTCCGCCGCACTATCGGTCTGTAAAGTTCGACCTTCGACGTATTAACGGCTTGCCT
TTGGCGCTGTGCAACCTGCGCGCTGGGGCTTGACCCTCCGAAGACAGTGCGGATCTCGTGTG
CGGCAAATGGTCGGGGTAAACACGACTTACATGTACCGCGGGGTACTATGGTAACGTATTT
GCATTTCCCTGCGGTAGTGTGCGCGCGGGGCATGATTAGCACCAGCTCGCTGGAATACACAGT
GGAGGAGGTTAAAAAGGCTAAAGCGCGCATGACGGGTGAGTATTTACGCTCTGTTGCGGAT
CTGATGGTGACGAAAGGTCGGCCGTTATATACGGTTGCGGGGAAGTATATTGTTAGCGATAC
AACGCGGGTGGGGTTTGACGCTATAGATTTTGGGTGGGGGAAGCCAGTATATGGGGGGCCT
GCGCGCGCTTTCCCGCTGATTAGTTTCTATGCGCGCTTTAAGAATAATCGCGGGGAAGATGG
GACCGTTGTATTAATTTGTCTGCCGGAAGCAGCGATGAAGCGCTTTCAGGATGAGCTGAAAA
AGATGACCGAAGAGCATGTTGACGGTCCGTTTGAATACAAATTAATTAAGGTGATGTCGAAG
TTATAA

Codon optimized DNA sequence of *E. coli* Fdh to the genome of *E. coli*

ATGAAGATAGTGCTGGTGTGTACGATGCTGGAAAGCATGCCGCAGACGAGGAGAAGTTAT
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GATATAATCATCACTACTCCTTTTCATCCAGCGTACATAACCAAAGAAAGAATCGACAAGGC
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TTAATCAAACGGGGAAGAAAATTTAGTCTGGAAGTAACAGGCTCTAATGTTGTCAGCGTT
GCGGAGCACGTACTGATGACCATGTTGGTTTTGGTGCCTAACTTCGTCCCGGCGCATGAACA
GATTATAAATCATGACTGGGAAGTTGCTGCCATAGCGAAGGATGCCTACGACATCGAGGGC
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AGGTCGGGGCTCGGAGAGTCGAAAATATCGAAGAACTTGTGCTCAGGCGGATATCGTCAC
GATAAATGCTCCACTTCACGCGGGCACTAAAGGGTTAATAAATAAAGAATTGTTATCGAAGT
TTAAGAAAGGTGCGTGGCTGGTAACACGGCGCGGGGCGCGATATGCGTAGCAGAAGACGT

AGCTGCTGCTTTAGAGTCGGGACAACTGAGAGGTTATGGGGGAGATGTATGGTTCCCCAAC
CAGCTCCAAAGGATCATCCTTGGCGGGATATGCGCAATAAGTACGGGGCAGGAAACGCAAT
GACCCCTCATTACAGCGGAACCACTCTTGATGCCAGACCCGCTACGCAGAAGGTACGAAGA
ATATTTTGAATCGTTTTTTACGGGCAAGTTTGACTACCGCCACAGGACATTATCTTTTAA
ACGGGGAATATATTACGAAGGCGTACGGGAAGCATGATAAGAAGTAA

Codon optimized DNA sequence of *E. coli* *FadD* to the genome of *E. coli*

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GCATGTTATTCTGACCCGTATGGGTGATCAGCTGAGTACCGCAAAAGGCACCGTTGTTAATT
TTGTGGTGAATACATTAAACGCCTGGTGCCGAAATATCATCTGCCTGATGCAATTAGCTTTC
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TCGTAATATGCTGGCCAATCTGGAACAGGTTAATGCAACCTATGGTCCGCTGCTGCATCCGG
GTAAAGAACTGGTTGTTACCGCACTGCCGCTGTATCATATCTTTGCACTGACCATTAAATTGCC
TGCTGTTTATTGAACTGGGTGGTCAGAACCTGCTGATTACCAATCCGCGTGATATCCGGGTC
TGGTTAAAGAGCTGGCAAAATATCCGTTTACCGCAATTACCGGTGTTAATACCCTGTTTAATG
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GGTGGTATGCCGGTGCAGCAGGTTGTTGAGAACGTTGGGTAAACTGACAGGTCAGTATCT
GCTGGAAGGTTATGGTCTGACCGAATGTGCACCGCTGGTTAGCGTTAATCCGTATGATATTG
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GCTGGGTTATTGGCAGCGTCCGGATGCAACCGATGAAATTATCAAAAATGGTTGGCTGCATA
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TCCTGGTGTTCAGAGGTTGCAGCAGTTGGTGTTCGAGCGGTAGCTCAGGTGAAGCAGTTA
AAATCTTCGTGGTTAAAAAAGATCCGAGCCTGACCGAAGAAAGCCTGGTTACCTTTTGTCTG
CGCCAGCTGACCGGTTATAAAGTTCCGAAACTGGTTGAATTTCTGTGATGAACTGCCGAAAAG
CAATGTGGGTAAAATTCTGCGTCGTGAACTGCGTGATGAAGCACGTGGTAAAGTTGATAATA
AAGCCTAA

Codon optimized DNA sequence of *C. palustris* *FatB1* to the genome of *E. coli*

ATGGTTGCAGCAGCAGCAAGCAGCGCATGTTTTCCGGTTCGAGTCCGGGTGCAAGCCCGAA
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TTTGTTCACCGGAAAAACGTTGGACCATGTTTGATCGTAAAAGCAAACGTCCGAATATGCT

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 GAATGGCGTCCGAAAAATGCAGGCACCAATGGTGCAATTAGCACCGGTAAAACCTAA

Codon optimized DNA sequence of *R. hybrida* AAT to the genome of *E. coli*

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Codon optimized DNA sequence of *O. basilicum* *GES* to the genome of *E. coli*

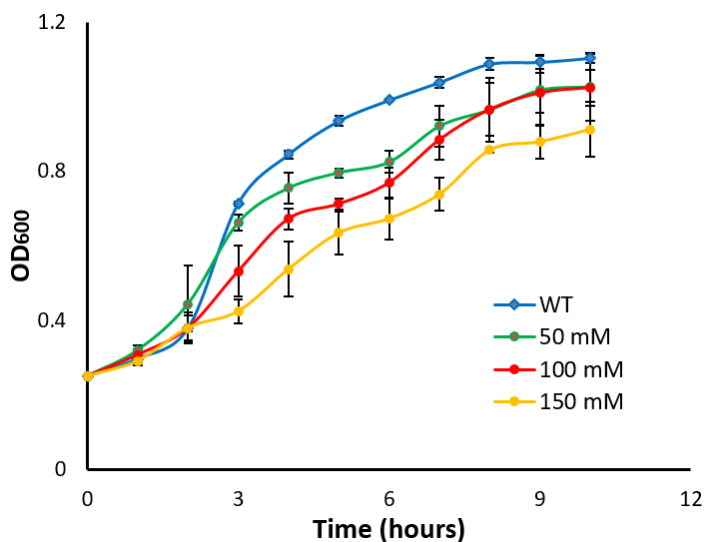
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GAAGAACAAGAACGCGGAGATCTGGCAAGCTGTGTTTCAGCTGTTTCATGAAAGAAAAAAGCC
TGACCGAAGAAGAAGCCCGTAGCCGTATCCTGGAAGAGATTAAAGGTCTGTGGCGTGATCT
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TGGCACGCGCAAGCCAGGTTGTGTATAAACATGATCAGGATACCTATTTACAGCAGCGTGAT
AATTATGTTGATGCCCTGTTTTTTACCCAGTAA

Supplementary Figure 1 Full length sequence of each gene used in this study that have been either codon harmonized or optimized to the genome of *E. coli*. Codon harmonization was carried out by hand using codon usage tables. Codon optimization was done using GeneArt software.

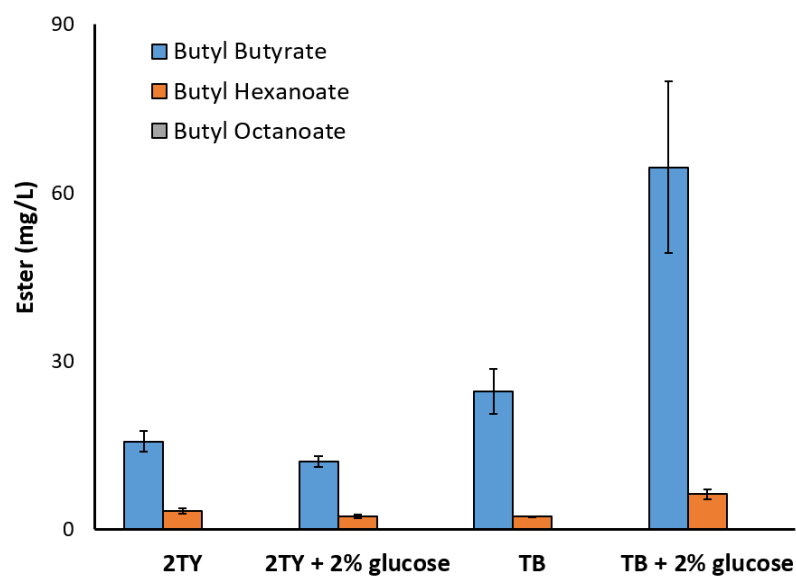
Supplementary Table 1 Oligonucleotide primers used in this study. All primers were synthesised and purchased from Eurofins Genomics (Germany).

Primer	Sequence
EcAAT_BamHI_For	AAAGGATCCGCGAGCTTTCCG
EcAAT_NSXhoI_Rev	AAACTCGAGTAACTTCGACATCACCTT
Cs2848_NdeI_For	AAACATATGGAATAATATAAACCTT
Cs2848_BamHI_Rev	AAACTCGAGTTTAAGAAATTTGAC
Cs4859_NdeI_For	AAACATATGTTAAGAAGAGTAAAA
Cs4859_BamHI_Rev	AAACTCGAGTAGATTCTTTTCTTT
Cs5680_NdeI_For	AAACATATGAATGGAATCATTGAA

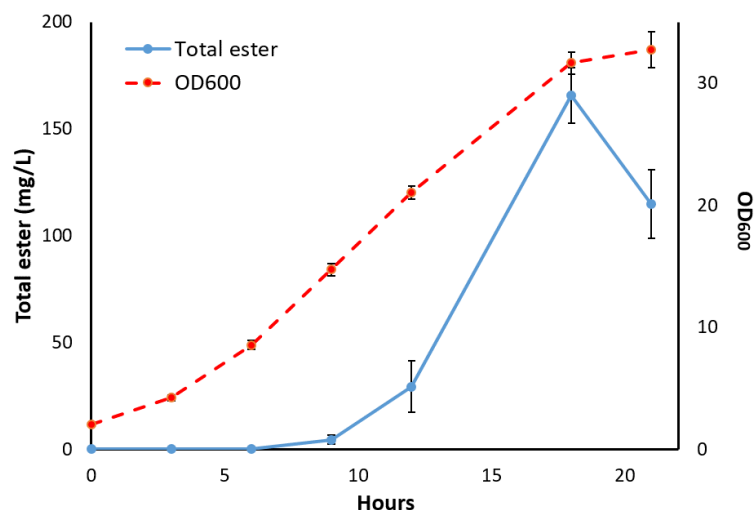
Cs5680_BamHI_Rev	AAACTCGAGAAGGTGCATTAACCA
Cs2944_NdeI_For	AAACATATGACAGAGTATATTA
Cs2944_BamHI_Rev	AAACTCGAGTTTTTAATTTTTTCCC
Cs2130_NdeI_For	AAACATATGTCGTTAAATCCTAAA
Cs2130_BamHI_Rev	AAACTCGAGTTTTATCTTATTGAA
Cs5781_NdeI_For	AAACATATGGCATTTTTACAAACA
Cs5781_BamHI_Rev	AAACTCGAGTTCGTTAATCCAAAG
Ca2917_NdeI_For	AAACATATGATAAAATAGGATTATT
Ca2971_BamHI_Rev	AAACTCGAGTCTCCAAAGATACT
Ca1962_NdeI_For	AAACATATGCAGGGGAGAGTTTAC
Ca1962_BamHI_Rev	AAACTCGAGTTTTTCATTAAGCCA
CaLipG_NdeI_For	AAACATATGGCACACGTAAATACA
CaLipG_BamHI_Rev	AAACTCGAGTCGCCAACATGAATG
SalRBS_For	GTCGACAGACATTAAAGAGGAGAATACT
Ter_For	AGG TAG AAA GGT TCG ACA
AATM94F_For	AATCGGAAGCTGTTTGTGGATTGTA
AATM94F_Rev	GTACAATCCACAAACAGCTTCCGA
AATS99G_For	GATTGTACAGGTGAAGGGGTGTTA
AATS99G_Rev	TAACACCCCTTCACCTGTACAATCC
AATL178F_For	GGTTAGTGCAGTTTCTTACCACC
AATL178F_Rev	GGTGGTAAGAACTGCACTAACCC
AATF185I_For	GCGGAGATTGCGCGG
AATF185I_Rev	CCGCGCAATCTCCGC
AATF203L_For	CGCGAGCTCCTCGCG
AATF203L_Rev	CGCGAGGAGCTCGCG
AATR246S_For	AAAGAGATAAGCGCAATTCGG
AATR246S_Rev	CCGAATTGCGCTTATCTCTTT
FdhInternal_For	AGCTCCAAAGGATCATCCTT
FATBinternal_For	GAATATCGTCGTGAATGTGGT
T7_For	TAATACGACTCACTATAGGG
T7_Rev	CTAGTTATTGCTCAGCGGT
TerFdhXhoI_For	TTTGTTAGCAGCCGGATC
TerFdhSal_Rev	AATACAAATTAATTAAGGTGATGTCG
FadDSac_For	AAAGAGCTCTTTGTTTAACTTTAAGA
FadDNot_Rev	TTTGCGGCCGCTTAGGCTTTATTATCA
FATBSalI_For	AAAGTCGACTTTGTTTAACTTTAA
FATBSacI_Rev	TTTGAGCTCTTAGGTTTTACCGGTGC
FadEupstream	GGACTTTTTGACCTGAAGTGC
FadEdownstream	ACCTGGCTGAATGTGAACG
RhAATBamHI_For	AAAGGATCCATGGAGAAAATTGAGGTCAGT
RhAATXhoI_Rev	TTTCTCGAGTTAATCCATACCAACTGAAGA
Gibson primers	
Hyg_For	GAGTAAACTTGGTCTGACAGTTATTCCTTTGCCCTCGGAC
Hyg_Rev	GTGAGTTCAGGCTTTTTACCCATACTCTTCCTTTTCAATATT
pBESTBB_For	AGTTCAGGCTTTTTACCCATACTCTTCCTTTTCAATATTATTGAAG
pBESTBB_Rev	GTCCGAGGGCAAAGGAATAACTG TCAGACCAAGTTTACTC
HygP15A_For	TGAGCGCAACGCAATTAATGACCGATGCCCTGGAGAG
HygP15A_Rev	ATGCTTCAATAATATTGAAAAAGGAAGAGTATGGGTAAAAAGCTCT
Pet21BB_For	AGTTCAGGCTTTTTACCCAATACTCTTCCTTTTCAATATTATTGA
Pet21BB_Rev	AGGCTCTCAAGGGCATCGGTCATTAATTGCGTTGCGCTC
GDSGibson_For	GTTCTGTGATTGCCGCAT
GSGibson_Rev	TTTATTTGATGCCTGGAGATCCTTACTCGAGTTTGGATCCTTACT
	GGGTAAAAAACAGGGC
qRT-PCR primers	
Ter4_For	GTCATGGGTGGAGAGGACTG
Ter4_Rev	TGCTTGGGTCTCCTTGTTT
AAT2_For	AGCTTTCGCTTCGTTAGT
AAT2_Rev	TCGACAGTTTGCCTTGTA
RrsA3_For	CTCAACCTGGGAACGTCATC
RrsA3_Rev	ACCTGAGCGTCAGTCTTCGT



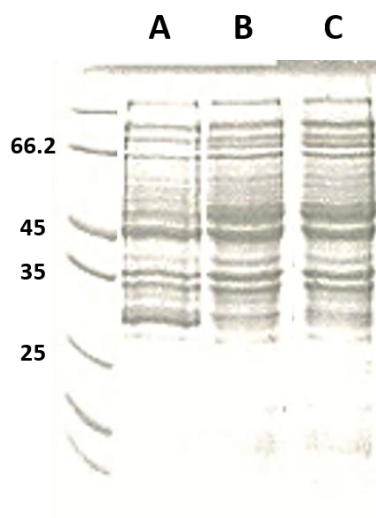
Supplementary Figure 2 Growth rate of wild type DH5α *E. coli* exogenously fed 0 mM, 50 mM, 100 mM, and 150 mM of butyl benzoate. Cultures were grown at 37°C and 250 rpm, and the OD₆₀₀ was taken every 30 minutes until stationary phase was reached. Butyl ester was fed 1.5 hours after first reading. Data are the mean ± standard deviation from three biological replicates.



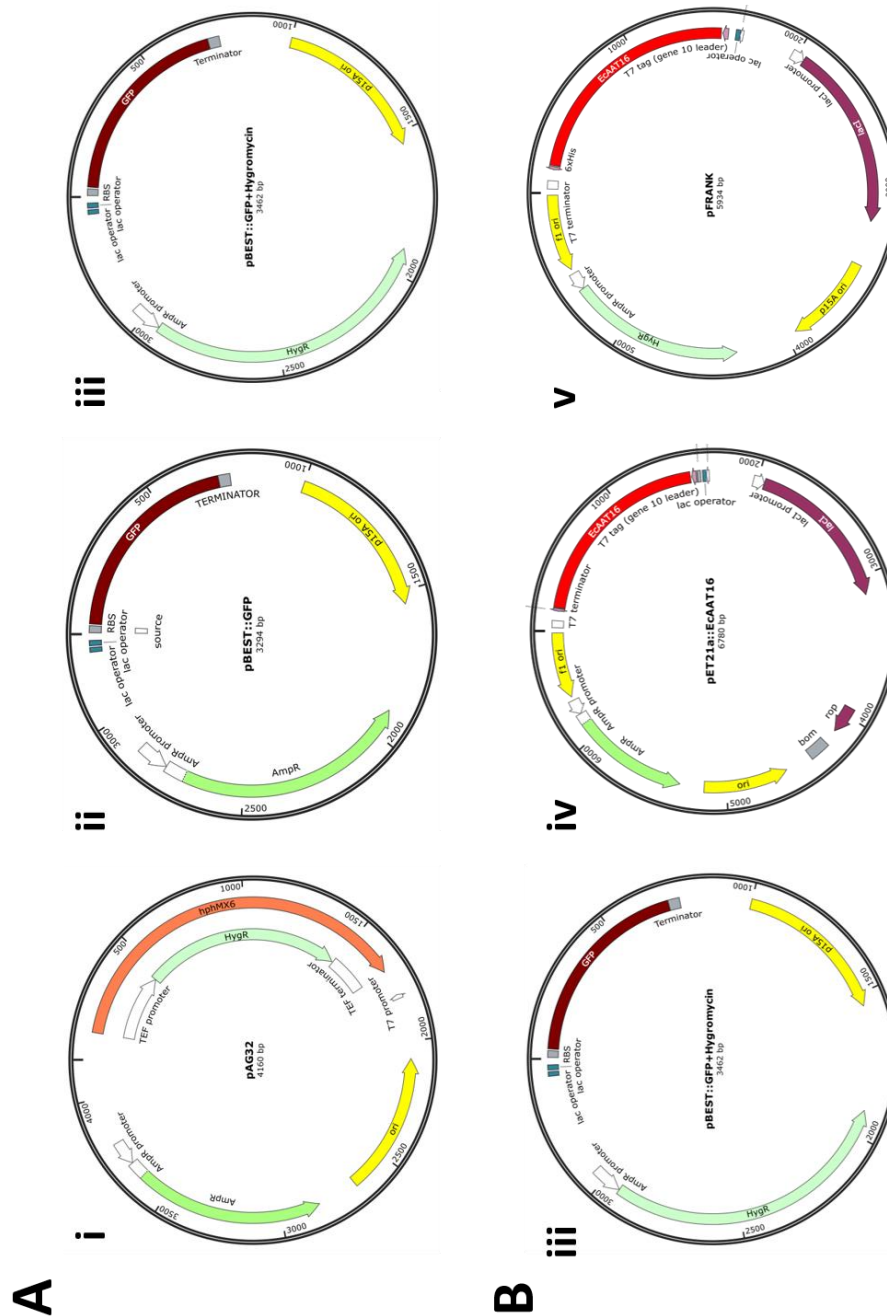
Supplementary Figure 3 Quantification of butyl esters present in the supernatant of cultures of *E. coli* strain C43 (DE3) harbouring pET21a::EcAAT incubated in different growth medias. Culture supernatants were analysed after growth at 20°C for 21 hours after induction with 0.4 mM IPTG. Cultures were grown in either 2TY +/- 2% glucose media or TB +/- 2% glucose media. Data are the mean ± standard deviation from three biological replicates.



Supplementary Figure 4 Time course of butyl ester production from pET21a::*EcAAT*. Cultures were grown in TB+2% glucose at 20°C and 250 rpm. Cultures were induced with 0.4 mM IPTG and incubated for 2 hours before the addition of 10 mM butanol and 5 mM octanoic acid. Lipid analysis was carried out at 3, 6, 9, 12, 18, and 21 hours. Data are the mean \pm standard deviation from four biological replicates.



Supplementary Figure 5 SDS-PAGE gel showing of the soluble protein fraction of **A** *E. coli* strain C43 (DE3), **B** *E. coli* strain C43 (DE3) harbouring pET21a::*EcAAT* without induction, and **C** *E. coli* C43 (DE3) harbouring pET21a::*EcAAT* with induction. Cultures were incubated at 37°C until OD₆₀₀ reached 0.8, and then incubated at 20°C for 18 hours before analysis. The culture of C43 (DE3) harbouring pET21a::*EcAAT* run in lane C was induced with 0.4 mM IPTG at OD₆₀₀ 0.8. The *EcAAT* protein has a molecular weight of 51 kDa.



Supplementary Figure 6 Plasmid maps showing the creation of pFRANK. The Hygromycin resistance gene from pAG32 (i) was inserted in the place of the Ampicillin resistance gene of pBEST::GFP (ii) to create pBEST::GFP+Hygromycin (iii). This was done using Gibson assembly where the Hygromycin gene from pAG32 and the pBEST::GFP backbone were amplified with complementary overhangs before assembly. In the second step, the Hygromycin gene and p15A ori region of pBEST::GFP+Hygromycin (iii) was inserted in the place of the Ampicillin resistance gene and pColE1 ori region of pET21a::EcaAT16 (iv) to create pFRANK (v). This was done using Gibson assembly where each respective region was amplified with overhangs and assembled.

AeAAT 1 ----MASSV---RLVKKEVLVAVDPTPTVLSL--SSLDLSQLFLRFPEYLLVVASPH-G
 Ban-AAT 1 -----MSFAVTRTSRSLVTPCGVTPPGSLGL--SAIDRVPGLRHMRSLHVT-----
 MpAAT1 1 ----MMSFSVLQVKRLQPELITPAKSTPQETKFL--SDIDDQESLRVQIPIIMCYKDNPSL
 AcAAT 1 -MASFPSSLVFTVRENEPTLVLPSSKSTPRELKQL--SDIDDQEGELRFQVPMVIMFYKRKL-S
 CmAAT1 1 -----DFSFHVRKCCPELIAPANPTPYEFKQL--SDVDDQQSRLQLPEVNIYPHNP-S
 CmAAT2 1 METMQTIDFSFQVRKCCPELIAPANPTPYEFKQL--SDVDDQQSRLRFQPLVNIYHNP-S
 VpAAT 1 -MAEKASSLMFNVRHEPELITPAKSTPRETKLL--SDIDDQDGLRFQVPIIQFYKNN-S
 CmAAT3 1 ----MASSLVFQVQSSQPLIPPSDPTPEFKQL--SDIDDQEGELRFQIPVPIQFYRHDP-R
 BEBT 1 --MAHDQSLSFQVCRKPELIRPAKQTPHEFKKL--SDVEDQEGELRFQIPVPIQFYKHNNES
 BEAT 1 -----MNVTMHSSKLLKPSIPTPNHLQKLNLSLTDQIQLPFFYVGLIFFHYETLSDN
 CmAAT4 1 -----MEVKVLSKETIIPSSPTPHIQPLNLSLDQLSPMLYIPLLLFYPMKKSY
 RhaAT 1 -----MEKIEVSIISRITIKPSAAS--SSLHPYKLSITDQFTPTTYFPVIFFFYPITDRV
 SAAT 1 -----MEKIEVSINSKHTIKPSTSS--TPIQPYKLTLLDQLTPPAYVPIVFFYPITDHD
 VAAT 1 -----MEKIEVSIISKHTIKPSTSS--SPLQPYKLTLLDQLTPPSYVPMVFFYPITGPA

AeAAT 52 VD--RAVTAARVKAALARSLVPYYPLAGRVKTRD-STGLDVVCOAQAGLLEAVSDYLA
 Ban-AAT 46 RQ--GREFARIIREALSALVKYYFAGRFVLDPEGGEVVRVACTGEGAWFEAKADCSL
 MpAAT1 56 NK--NRNPVKAIREALSRALVYYYPLAGRLREGF--NRKLVVDNNGEGILFEVASADVIL
 AcAAT 58 ME--GEDPVKVIKEALAEALVFYYFAGRLIEGF--NRKLMVDCTSEGVLFEADADIEV
 CmAAT1 52 LE--GRDPVKVIKEATGKALVFYYPLAGRLREGF--GRKLFVECTGEGILFEADADVSL
 CmAAT2 59 LE--GRDPVKVIKEATAKALVFYYPLAGRLREGF--GRKLFVECTGEGILFEADADVSL
 VpAAT 58 MQ--GKNPAKLIKSALETLVHHYPLAGRLREGF--GRKLMVECTGEGILFEADADVIL
 CmAAT3 55 MA--GTDPAKVIKEATAKALVFYYFAGRLREGF--GRKLFVECTGEGVMFEADADVSL
 BEBT 58 MQ--ERDPVQVIREGIARALVYYFAGRLREVD--GRKLVVECTGEGVMFEADADVIL
 BEAT 51 ----SDITLSKLESSLSETITLYYHAGRYN----GTDCVIECNQCGICGVETAFDVEL
 CmAAT4 51 QHQDHNAATIKTSLSKTISRFLLAGRLI----GK-S-THCNDKGAVEMEATINSNM
 RhaAT 53 FN--LPQTTDLKNTVSCALTLYHPLSGRLK----NN-ILYIDDFEAGVPYLEARVNFM
 SAAT 53 FN--LPQTTADLRQALSETITLYYPLSGRVK----NN-ILYIDDFEAGVPYLEARVNCDM
 VAAT 53 VF--NLQTTADLRHALSETITLYYPLSGRVK----NN-ILYIDDFEAGVPYLEARVNCDM

AeAAT 109 SDFQRA--PRSVTEWRKLILVE----V---FKVVEPLVVQLTWLSDGCVALGVGFSHCV
 Ban-AAT 104 EDVKYLDPL--MIPEDALPKPCPLN---PLDLP-LMLQVTEFVGGGFVVGILSVHTI
 MpAAT1 112 EQL-GDKLLEPCPLEE-FLYNFEGSDG---IIDCPLLLIQVTRCLTCGGFILAIRINHMT
 AcAAT 114 NQLIGDTHDEGFSYLDE-LTHDVEGSEG---IIGCPLLLIQVTRFRCCGMAFAIRINHMT
 CmAAT1 108 EEF-WDTLPYSLSSQNNIITHNALNSDE---VINSPLLLIQVTRCLKCGGTFGLCFNHMT
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 VpAAT 114 HEF-GDDEPFPFCLVE-LLYDVEGSSG---IIDTPLLIIQVTRCLKCGGTFFAIRINHMT
 CmAAT3 111 EQF-GDALQFPFCLVE-PLFDVENSNG---VIDCPLLLIQVTRCLKCGGTFFAIRINHMT
 BEBT 114 EQF-GDALQFPFCLFDQ-LFDVVEGSSG---IIDSPLLLIQVTRCLKCGSFFFAIRINHMT
 BEAT 102 HQFLLG---EESNNLDL-LVGLSGFLSE---TETPELAATQINMFKCGGLVIGAQFNHII
 CmAAT4 104 FDLKE---ENNEVITK-LIPCSLLCN-TKPIEEYFQIVVQANIFECGGAISCLLHKL
 RhaAT 105 IDFLRL---EKIEWINE-FVPMAYRKETIS-EFLPLLGIOVNIFDS-GIAGVSFSHKI
 SAAT 105 TDFLRL---RKIEQINE-FVPIKEFSMEAISDERYPILGWQVNVFDS-GIAGVSFSHKI
 VAAT 105 NDFLRL---EKIEQINE-FVPIKEFSMEAISDERYPILGWQVNIENS-GIAGVSFSHKI

AeAAT 159 IDGISSEFINLFAELATCARLSEFQ----PKPVWDRH--LINSAGRNI-GTH---P
 Ban-AAT 158 ADGLGVVQFINAVAEIARGLP-K--PT----VEEAWRE--VLPN--PEKIPPGG---P
 MpAAT1 167 CDAAGLLFTTATAEMARGAH-A--PS----ILPVWERE--LLEARDPERITCAH---H
 AcAAT 170 SDAPCLVQLLTIAEFARGAEGA--PS----VPPVWORE--FLAARQPESTITFQH---Y
 CmAAT1 164 ADGEGIVQFMKATAETARGAF-A--PS----ILPVWQRA--LITARDPERITFHH---H
 CmAAT2 171 ADGEGIAQFMKATAETARGAF-A--PS----ILPVWQRA--LITARDPERITFHH---Y
 VpAAT 169 SDASGLVQFMMAVGEMARGOR-S--LS----IOPVWERH--LLEARDPERITFHH---H
 CmAAT3 166 SDASGLVQFMMAVGEMARGAT-A--PS----VPPVWQRA--LLEARDPEKVTCHH---R
 BEBT 169 ADAAGIVLFMAVGEMARGAA-T--PS----TLPVWDRH--LLEARDPEKVTCHH---R
 BEAT 155 GDMFTMSTFMSWAKACRVCIK-----EVAHETEGELAP-LMPSAK-----
 CmAAT4 159 IDAATFCCFLRSWATTNRELLSLDHSPPNNNMVCDYKSFSSLPQTN--LLEPFLQSLIN
 RhaAT 159 NDGQTASCFKLSWAVFRGYRN-----KIIHENLSQALLFPFRD--DLPFK-----
 SAAT 160 IDGQTADCFKLSWCAVFRGCRE-----NIIHENLSQALLFPFRD--DLPFK-----
 VAAT 160 IDGRTSDCFKLSWCAVFRGSRD-----KIIHENLSQALLFPFRD--DLPFK-----

AeAAT 208 EFGRVPLDSGFV-TRFTQERISPTISITFDKTIWKELKNIAMSTSQPG---E--FPYISFE
 Ban-AAT 203 P-----V---FPSFKLIHATVDLSPDHTDHVKS-----RHLE---LTGQRCSTFD
 MpAAT1 214 EYEDVLIHSDGSGYASSNQSNMVQSRFFYFGAKEMRVLRK-----QIEP---HLISTCSTFD
 AcAAT 218 EYEQVINTT--T---DDNKSMTKSEFFGPKETRAIRS-----HFP--HYRSVSTFD
 CmAAT1 211 EYDQVVDKMSGL---IPVNSKIDQLFFFSQLQISTLRQ-----TLPA---HLHDCPS-FE
 CmAAT2 218 EYDQVVDTKSTL---IPANNMIDRLFFFTQRCISTLRQ-----TLPA---HLHDCSS-FE
 VpAAT 216 EYDDEEDTKGTI---IPLDDMVHRSFFFCPSEMAAIRR-----LVPA---HFRS-ITSE
 CmAAT3 213 EYDEVVDTKGTI---IPLDDMAHRSFFFCPSEISAIRK-----ALPS---HLRQCSS-FE
 BEBT 216 EYEBVKGT--IF---TPFDDLHRSFFFCSTETISAIRK-----QIEP---HLRSCSTTIE
 BEAT 194 -VLNIPPP-----PSFEGVKFVSIRFVFHENALTRLRKEATEEDGDGDDQKKRPSRV
 CmAAT4 217 NDKAVVPP-----SSIFNRIRRFQRFERSEALDLKAKAKSC-----DIPNPTCVE
 RhAAT 204 -YVAMMER-----MWFGEKVVTRRFVDAKAISALQDEGKSE-----YVPKPSRVQ
 SAAT 205 -YVDQMEA-----LWFAGKVVATRRFVFGKATSSIQDEAKSE-----SVPKPSRVH
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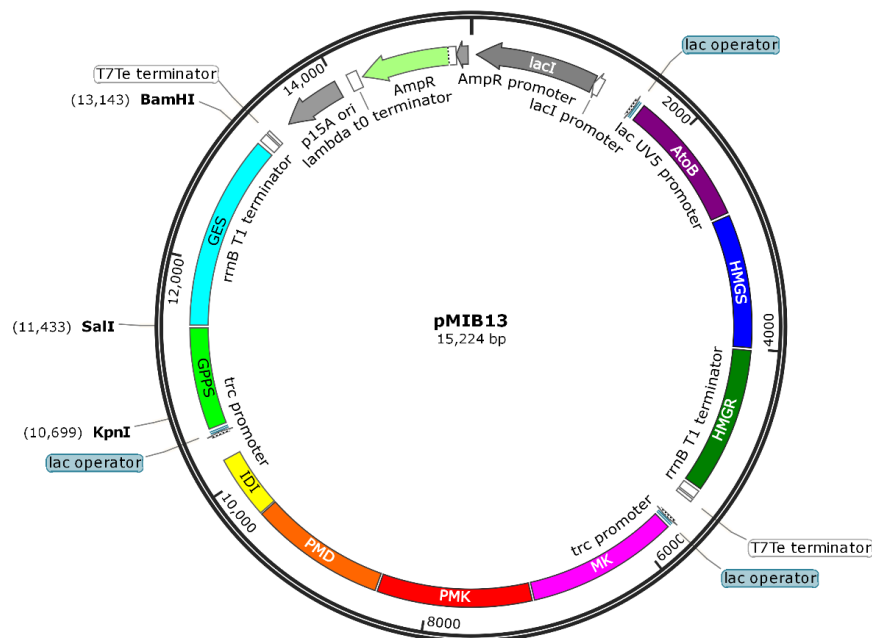
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 MpAAT1 266 LITACLWKRORTIALNLN---PKEAVRVSCIVNARCKHN---VRIPLCYGYNAAFAPA
 AcAAT 265 VLTACLWRORTIALGLD---PKTVRUSCAANGRGKHD---LHVRGYYGNVFAFA
 CmAAT1 259 VLTAVVWRORTIALQFK---PEEEVRFLCVNLRSKID---I--PLGYYGNVAVVFA
 CmAAT2 266 VLAAYVWRORTIAFOLK---PEEEVRFLCVNLRSKID---I--PLGYYGNVIVFA
 VpAAT 264 VLTAVLWRCYTIALQPD---PEEEMRVICVNSRRTKLN---PPLPTCFYGNCIAFA
 CmAAT3 261 VLTACLWRORTISLQPD---PEEEVRFLCIVNSRSKEN---PPLPTGYGYNAAFAPV
 BEBT 263 VLTACLWRORTIALKPN---PEEEVRMCTIVNARSKEN---PPLPDGYGYNAAFAPA
 BEAT 248 LVTAELSKSLIEMDCAPK-ELTKSRPSLMVHMMNLKRRTKL---ALE-NDVSGNFFIVVN
 CmAAT4 264 TLTCFIVKYLKVVADDDG---SQRPSTSHVVNTRKML-E---PSLGEVSLGNIMWGTV
 RhAAT 250 ALTGELWKHQLAASRALSSGT-STRFSVASQTVNLRSKNM---KTLTDNAIGNIFLWAS
 SAAT 251 AVTGELWKHLIAASRALTSGTTSTRLSIAQAVNLRTRNM---ETVLDNATGNLFWMAQ
 VAAT 251 AVTSELWKHLIATSRALTSGTTSTRLSIATQVNVNRSRNM---ETVWDNAIGNLIWFAP

AeAAT 313 AQ---TSVKDLTEKGLGYCADLVRGAKERVGDYAREVVESVSWP-----R
 Ban-AAT 297 AT---APSGRIASAEILIDVVSIIIRDAKSRIPGEFAKWAAGDFKDD-----P
 MpAAT1 318 AI---SKAEPLCKNPLGYALELVRKAKATMNEEYLRVADLLVLR-----G
 AcAAT 316 VV---SRAGMISTSSLEYTVEEVKKAKARMTGEYLRVADLMVTK-----G
 CmAAT1 308 VI---TTAAKLCGNPLGYAVDLIRKAKAKATMEYIKSTVDLMVIK-----G
 CmAAT2 315 VI---TTVAKLCGNPLGYAVDLIRKAKAKATKEYIKSTVDFMVIK-----G
 VpAAT 315 AI---SQAKKICENPFGYTLQLVKQTKVDVTEEYMRSAADLMAMK-----G
 CmAAT3 312 AI---TTAGKLCQNPLGYALELVRKAKADVTEYMKSVADLMVIK-----G
 BEBT 314 AV---TTAGKLCNNPLGFALELIRKAKREVTEEYMHSAADLMVAT-----G
 BEAT 303 AESK-ITVAPKITDL-----TESLGSACGEITSEVAKVDDAEVSSMVLNSVREFY
 CmAAT4 316 AHHFSTTRNEEFEGLELSKLVSLIRQSFKINKDYIKEIMGGDKER-RRGVMKLUGE-I
 RhAAT 306 ARLDLNDTAPGSSDLKLCDLVNLNENSIKEFNSDYLEIKGKEGYG-GMCDLLDFMEEGS
 SAAT 308 AILELSHTTPEISDLKLCDLVNLNENSVKQCNGDYFETFKGKEGYG-RMCEYLDFORTMS
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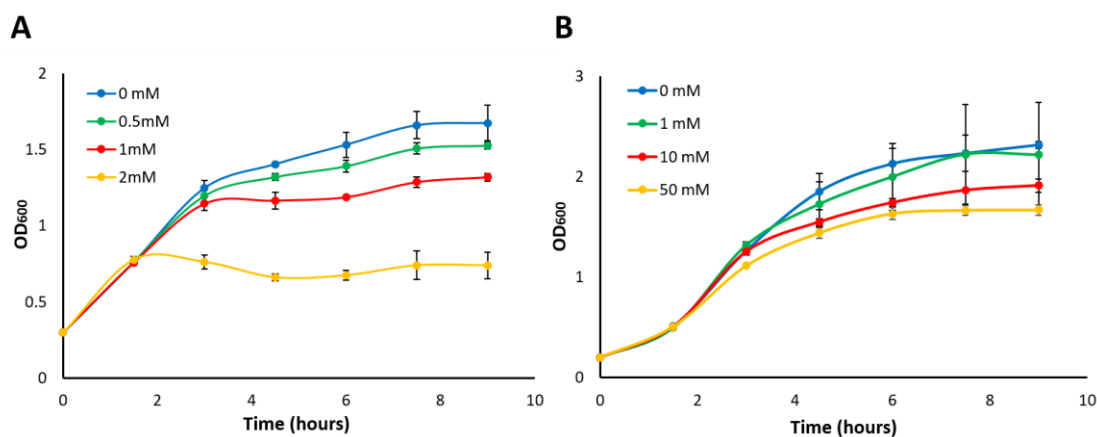
AeAAT 356 RASPDVGVLI-ISQWSRLGLDRVDFGLGRP--VQVGPICC-----DRYCLFIPVR-
 Ban-AAT 340 Y-ELSFYNSLIFVSDWTRIGFLDVMGWGKPLHVIFPAYLDIMAVGIIG-----APPA-
 MpAAT1 361 RQKYSTGSYLIVSDNTRVGGEDVNEFGWGO--PVFAGEV--KA-----LDLISFYVQHK
 AcAAT 359 RPLVTVAGNYI-VSDITRVGFDAIDFGWGK--PVYGGPA--RA-----FPLISFYARFKN
 CmAAT1 351 RPYFTVVGSEFM-MSDLTRIGVENVDFGWGK--AIFGGPT--TTGARITRGLVSECVPFMN
 CmAAT2 358 RPRFTEIGPFM-MSDLTRIGFENVDFGWGK--AIFGGPI--IGGCGIIRGMISSTAFMN
 VpAAT 358 RPHFTVVRRYM-VSDVTRAGFGLVDFGWGKPEPVYGGPA--KGGVGPIPGVTSFFVPFKN
 CmAAT3 355 RPHFTVVRTYL-VSDVTRAGFEDVDFGWGK--AMYGGPA--KGGVGAI PGVASFYIPFKN
 BEBT 357 RPHFTVNTYL-VSDVTRAGFGEVDFGWGE--AVYGGPA--KGGVGVI PGVTSFYIPLRN
 BEAT 353 YEWGKGEKNVFVYSMCRFPLYEVDFGWGLPSLV---DTT-----AFP--FGLIVLMDE
 CmAAT4 374 NKWP--ISNLYFFTSWKNKLNEVDFGWGKPLWS--AIA-----GDPNEMMGNIIVLVD
 RhAAT 365 FVEP--APEYSFSSWTRF-FDQVDFGWGRPSWV--GFS-----GRVETR--NETIFVE
 SAAT 367 SMEP--APDIYLFSSWTNF-FNPIDFGWGRTSWV--GVA-----GKIESASCKETILVP

VAAT	367	SMEP--APEIYLF S WTFN F - F NQ L D F GW G F T S W I -- G V A ----- G K I ESAF C N L T T L V P
AeAAT	404	-D-RTES V K M V A V ETSA V DR Y E Y F I RS P YS-----
Ban-AAT	392	-P-QKGTR V MA Q C V E K EH M QA F LE E M K G F A-----
MpAAT1	412	-N-TED G I L V P MC L ESS A M R F Q Q E L I ER I T Q EP K E-D I C N -----N I R S T S Q-----
AcAAT	409	NR- G ED G T V VL I C L EE A M K R F Q D EL K M T EEH-- V D G P F E Y K L K V M S K L-----
CmAAT1	406	RN- G E K G T A S I C LE P P A M E R F R A N V H A S L Q V K Q V V DA V D S H M Q-T I Q S A S K-----
CmAAT2	413	RN- G E K G I V V P I C L EP P A M E R F R A N V H A S L Q V I Q V L D K V D R D M Q-T I L S A L -----
VpAAT	415	RK- G E K G I V V P T CL E TP A M E R F A K L M NE I L Q N L L V SA E -EN K S V F I V S A I -----
CmAAT3	410	KK- G E R G I V V P I C L EP A M E R F V K EL D ALL K AG K T I D G V D N K K P I F I A S A L -----
BEBT	412	RQ- G E K G I V V P I C L ES A AM E IE A EL N N T L N G K E- I E I A K -----H F T Q S S L-----
BEAT	402	APAC G G - T A V R A C I SE H D I Q F Q Q H H Q-----L I S Y V S -----
CmAAT4	424	NVLD G STE A W I L D E K E M Q L LE Q I P Q----- F LE F ALL N P S I N I PH N Q K T A D E I F S N
RhAAT	412	TQCD G - T DA W V T D E K Q M AM L EQ T P Q ----- F LA F AS P N P R I S I A S SV G M D -----
SAAT	416	TQC S G I - T E A W N T EE E K M AM L EQ T PH----- F LA L AS P K T L I -----
VAAT	416	TPCD T G I - T E A W N T EE E K M AM L EQ T P Q ----- F LA L AS P K T L I S R Y-----
AeAAT	---	
Ban-AAT	---	
MpAAT1	---	
AcAAT	---	
CmAAT1	---	
CmAAT2	---	
VpAAT	---	
CmAAT3	---	
BEBT	---	
BEAT	---	
CmAAT4	477	KLI
RhAAT	---	
SAAT	---	
VAAT	---	

Supplementary Figure 7 Amino acid sequence alignment of fruit or flower derived AAT enzymes from a number of different plant species. Sequences correspond to Genbank accession numbers: Ae (*Actinidia eriantha*) AAT (HO772637); Ban (*Musa sapientum*) AAT (AW025506); Mp (*Malus pumila*) AAT (AY707098); Ac (*Actinidia chinensis*) AAT (HO772640); Cm (*Cucumis melo*) AAT1 (CAA94432), AAT2 (AAL77060), AAT3 (AAW51125), AAT4 (AWW51126); Vp (*Vasconcellea pubescens*) AAT (FJ548611); Cb (*Clarkia breweri*) BEBT (AAN09796), BEAT (AAF04787); Rh (*Rosa hybrid*) AAT (AAW31948); Fa (*Fragaria x ananassa*) SAAT (AAG13130); Fv (*Fragaria vesca*) VAAT (AAN07090). Sequences were aligned using Clustal Omega and BoxShade. Residues of AcAAT that were mutated are highlighted in yellow.



Supplementary Figure 8 Plasmid map of pMIB13 modified from Alonso-Gutierrez *et al.*, (2013) with the substitution of a terminal limonene synthase (*LS*) gene for a geraniol synthase gene (*GES*). Map shows the location of the original *KpnI* and *BamHI* restriction sites, as well as the newly inserted *SalI* restriction site.



Supplementary Figure 9 Wild type C43 (DE3) *E. coli* exogenously fed either geraniol (**A**) or geranyl acetate (**B**). Geraniol was exogenously fed into cultures to a final concentration of 0 mM, 0.5 mM, 1 mM, or 2 mM; while geranyl acetate was fed into cultures to a final concentration of 0 mM, 1 mM, 10 mM, or 50 mM. Cultures were grown at 37°C and 250 rpm, and the OD₆₀₀ was taken every 30 minutes until stationary phase was reached. Geraniol or geranyl acetate was fed 1.5 hours after first reading. Data are the mean \pm standard deviation from three biological replicates.

A – Primary investigations of into the viability of producing commercially valuable butyl esters in clostridial species *C. acetobutylicum* and *C. saccharoperbutylacetonicum*

A.1 Introduction

Clostridia are a diverse spore-forming gram positive genus of bacteria that are typically strict anaerobes; over the last two centuries they have been employed in a number of biotechnological processes – including the industrial production of secreted toxins for therapeutics and pharmacological agents from such species as *C. perfringens*, *C. botulinum* and *C. tetani* species, cancer therapy agents from *C. histolyticum* and *C. oncolyticum*, as well as alcohols and organic acids for use as biofuels and commercial product additives from *C. acetobutylicum* and *C. beijerinckii* (Jones and Woods, 1986; Schlechte and Elbe, 1988; Rood *et al.*, 1997; Gheshlaghi *et al.*, 2009).

The latter solventogenic species were first exploited for their fermentation of alcohols and acids during World War I as the acetone they produced was used for cordite manufacturing (Lütke-Eversloh and Bahl, 2011; Sauer, 2016). These clostridia naturally produce acetone, butanol and ethanol in a ratio of 3:6:1 through a process known as ABE (acetone-butanol-ethanol) fermentation (Figure A.1). A distinguishing characteristic of this clostridial solvent production is that it is a biphasic fermentation. The first phase is termed the ‘acidogenic’ phase, which occurs during exponential growth and involves the production and secretion of acetate, butyrate, carbon dioxide and hydrogen as the major products (Grupe *et al.*, 1992; Amador-Noguez *et al.*, 2011). The second phase is termed the ‘solventogenic’ phase, which is believed to be in-part initiated by the drop in culture pH and involves a halt to culture growth and the re-assimilation of the previously made acids for use as co-substrates for the synthesis of the major products, acetone, butanol and ethanol (Jones and Woods, 1986; Gheshlaghi *et al.*, 2009; Branduardi *et al.*, 2014).

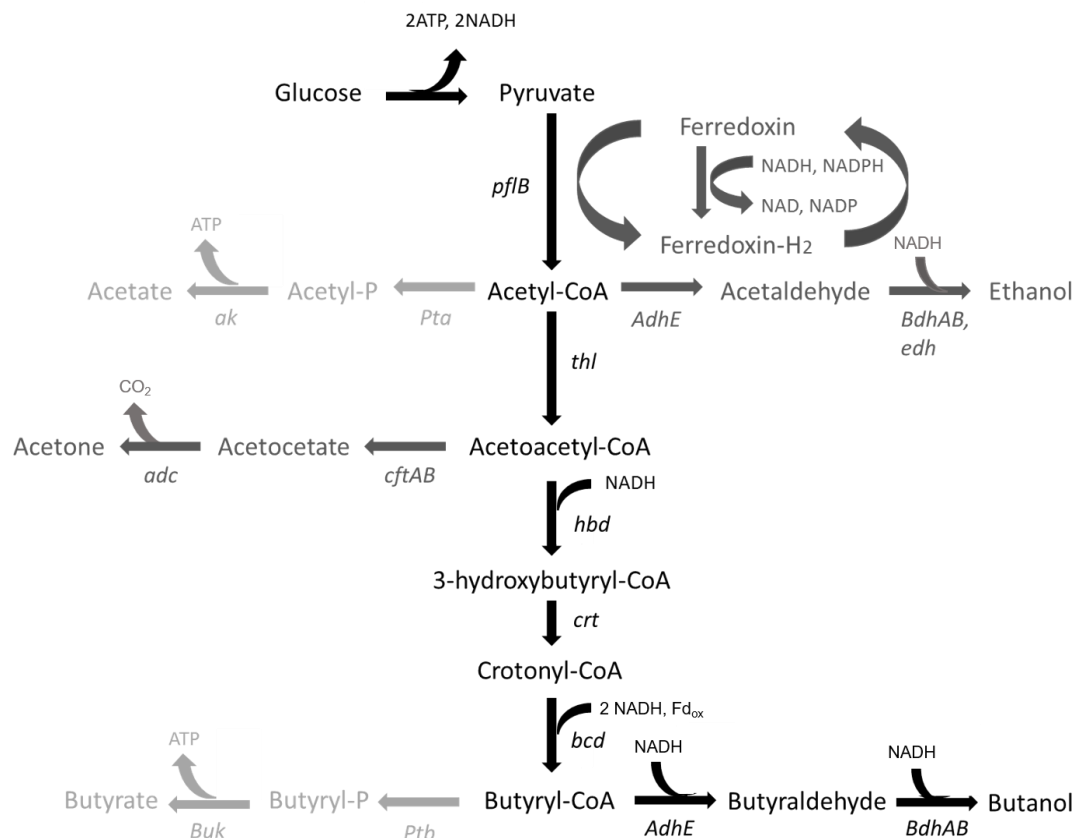


Figure A. 1 ABE fermentation pathway from *C. acetobutylicum*. Reactions leading towards n-butanol production are in black, while reactions leading to acetone and ethanol production are in dark grey. The acidogenesis reactions leading towards acetate and butyrate production are in light grey. Enzymes responsible for each reaction are indicated by italic letters next to each arrow.

Utilization of the second solventogenic phase of clostridial ABE fermentation for the purpose of n-butanol (normal butanol) production became popular after the war, as it was used to produce lacquers for the automobile industry (Sauer, 2016). In more recent years, much interest has developed in exploiting butanol as a renewable fuel alternative to gasoline; with it having been described as a superior biofuel compared to the more traditionally used bio-ethanol (Dürre, 2007; Ezeji *et al.*, 2007; Papoutsakis, 2008). Additionally, butanol has become an important intermediate in a number of chemical industries; notably it is used for making amino resins, plastics and polymers, lubricants, and specialty solvents for multiple commercial preparations (Lee *et al.*, 2008; Lütke-Eversloh and Bahl, 2011). Butanol's multiple commercial applications,

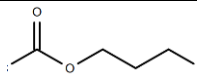
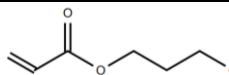
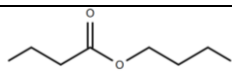
as well as the general growing interest in naturally sourced chemicals and fuels, has powered the development of clostridia as a production platform for butanol in biotechnology over the last few decades. Thus far, clostridial strain development has focused on optimizing fermentation techniques and downstream processing to maximize butanol production, as well as rational metabolic engineering strategies to minimize side product formation and also to improve upon solvent toxicity. The latter strategy, involving molecular manipulation, has grown in popularity in recent years as the portfolio of genetic tools available to clostridial species continues to grow (Ezeji *et al.*, 2004; Papoutsakis, 2008; Lütke-Eversloh and Bahl, 2011; Zue *et al.*, 2012; Branduardi *et al.*, 2014).

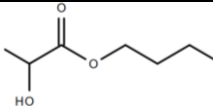
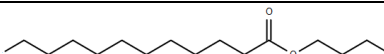
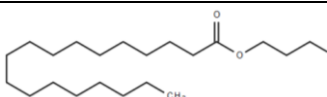
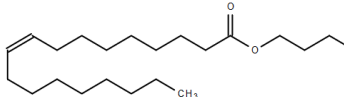
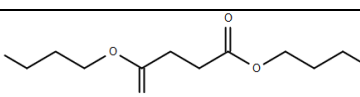
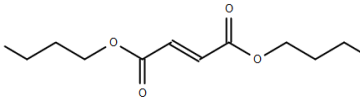
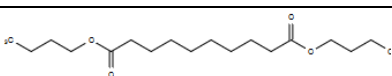
The microbial production of butanol derivatives for commercial products generally involves the extraction of butanol produced via ABE fermentation, followed by downstream chemistry to make such compounds as: butyl glycol ethers, butyl acrylates, butyl esters, 1-butene, etc. for a number of commercial applications (see above; <http://www.greenbiologics.com>). However, in recent years, as the genetic toolbox available to clostridial species has improved, interest in engineering strains for the production of these butanol derivative *in vivo* has become an attractive and lower cost option to the biotech industry. Butyl esters, specifically, have many commercial applications, including use as additives for flavour and fragrance products, cosmetics, and food, and as emulsifiers, emollients, and lubricants (Burdock, 1997; Rieger and Rhein, 1997; Schrader *et al.*, 2004; Winter, 2009). clostridia do not naturally produce butyl esters as an end-product, and to engineer a strain to do so would involve heterologous expression of an alcohol acyltransferase (AAT), which is an enzyme responsible for catalysing the esterification of a volatile alcohol to an acyl-CoA moiety to produce an ester (Salas, 2004; Souleyer *et al.*, 2005). AAT enzymes, which are generally found in plants, are responsible for synthesizing the ester compounds present in fruit and flower aroma. These enzymes display distinct substrate specificity for a number of alcohol and acyl-CoA substrates which varies depending on the enzyme in question (Olías *et al.*, 2002; El-Sharkawy *et al.*, 2005). Engineering clostridia for the production of esters has been attempted previously by several groups for acetate and butyrate esters (Horton *et al.*, 2003; Horton *et al.*, 2005; Van Den Berg *et al.*, 2013). This was first attempted for isoamyl acetate production in *C. acetobutylicum* by heterologous expression of the acetyltransferase 2 (ATF2) enzyme from *S. cerevisiae*. However even with excess substrate feeding, titres remained negligible (Horton *et al.*, 2003). In addition, significant degradation of isoamyl acetate was observed in culture feeding experiments, suggesting that this ester is being enzymatically broken down by endogenous esterase enzymes – a factor which may

be influencing the low product yields observed. More recent attempts at ester production – including butyl butyrate – in clostridia have also been met with low product titres, though no published work has further investigated the possibility of enzymatic breakdown of these products (Horton *et al.*, 2005; Van Den Berg *et al.*, 2013). To date, there is no available information on the prevalence of endogenous esterase or lipase enzymes capable of hydrolysing esters in solventogenic strains of clostridia.

In this work, the feasibility of using solventogenic clostridia as platform organisms for the production of commercially valuable butyl esters was investigated. To do this, three aims were outlined: (1) to investigate the potential microbial toxicity of a number of high value butyl and dibutyl esters (Table A.2) in two solventogenic clostridial species: *C. saccharoperbutylacetonicum* and *C. acetobutylicum*, (2) identify and characterise the chain-length substrate specificity of a number of putative esterases/lipases from each clostridial species, and (3) to determine whether or not any of our desired end-product esters are hydrolysed in culture.

Table A. 1 List of the butyl and dibutyl esters investigated in this work for possible microbial toxicity in clostridial strains *C. acetobutylicum* and *C. saccharoperbutylacetonicum*. Their chemical structure and commercial applications are listed above.

Ester	Structure	Application	Reference
Butyl acetate		Solvent, fragrance additive	Green Biologics – Cosmetics and person care formulation additives
Butyl acrylate		Adhesive, plastics	DOW Chemical Company
Butyl butyrate		Flavour and fragrance	PubChem

Butyl lactate		Solvent for cosmetics, flavour and fragrance	Green Biologics – Cosmetics and person care formulation additives
Butyl laurate		Flavour and fragrance	PubChem
Butyl stearate		Emollient/surfactant, lubricant, flavour/fragrance	Fine Organics
Butyl oleate		Emollient, lubricant	Green Biologics – Cosmetics and person care formulation additives
Dibutyl succinate		Fragrance and flavour	The Good Scents Company
Dibutyl maleate		Adhesive, plasticizer lubricant	Celanese Corporation – product description and handling guide
Dibutyl sebacate		Plasticizer for pharmaceuticals, lubricants for cosmetics and flavour	The Good Scents Company; Chemical Book

A.2 Results and discussion

A.2.1 Investigation of butyl and dibutyl ester toxicity in cultures of *Clostridium acetobutylicum* and *C. saccharoperbutylacetonicum*

To identify feasible butyl ester end-product for these clostridial production platforms the microbial toxicity of 10 commercially valuable potential ester candidates was first assessed. This was done as ideally our end-product(s) would have low to no toxicity in culture. These candidates included butyl and dibutyl esters that ranged both in acid chain length and saturation (Table A.1). To assess the toxicity of these candidates, cultures of *C. saccharoperbutylacetonicum* and *C. acetobutylicum* were exposed to a range of incrementally higher concentrations of a given ester and the effect on culture growth observed over the course of eight hours compared to a control with no added ester. This was done using the OD₆₀₀ as an indicator of culture health. Figures A.2 and A.3 show the results of these toxicity tests for *C. saccharoperbutylacetonicum* and *C. acetobutylicum*, respectively. From these growth curves, it can be seen that the patterns of toxicity are very similar between both clostridial strains, with the general trend being that butyl ester toxicity decreases as the chain length of the ester increases. The less polar longer chain esters such as butyl laurate, -stearate, -oleate, and dibutyl sebacate, exhibited no significant effect on growth rate compared to the wild type even at concentration up to 100 mM in culture (approximately 3-4% v/v in culture). Conversely, the shorter chain esters such as butyl acetate, -acrylate, and -butyrate, as well as the more polar long-chain esters dibutyl maleate and -succinate, displayed toxic effects beyond concentrations of 10 mM in culture (approximately 0.13-0.2% v/v in culture), with *C. saccharoperbutylacetonicum* being more susceptible than *C. acetobutylicum* at these lower concentrations. This is a general trend that has also been observed in *E. coli* and *S. cerevisiae* in work previously done by the Leak group (see chapter 3), and can be most likely attributed to the amphiphilic nature of these more polar esters. While the exact mode by which these compounds work to disrupt cell function is unknown, it has been hypothesized that it involves their incorporation with membrane lipids and subsequent disruption of essential function, and/or denaturation of crucial enzymes (Inoue *et al.*, 1991; Isken *et al.*, 1998; Mukhopadhyay, 2015). Often the LogP_(ow) value, which is the logarithm of the partition coefficient of a compound between octanol and water, is a good predictor of a compound's microbial toxicity, with values lower than 3.4-3.8 predicting high cellular toxicity (Inoue *et al.*, 1991). The results obtained here correlate with this LogP_(ow) toxicity prediction as we observed that butyl acetate, -acrylate, -butyrate, dibutyl maleate, and dibutyl succinate –

which all have $\text{LogP}_{(\text{ow})}$ values near or below 3 – exhibited high microbial toxicity, while all other esters tested – which have $\text{LogP}_{(\text{ow})}$ values above 4 – exhibited low microbial toxicity (Table A.2). Interestingly, butyl lactate, which has a $\text{LogP}_{(\text{ow})}$ value of 1.16, was well tolerated by both clostridial strains, and only began to cause a notable reduction in growth rate when present at a concentration of 50 mM in culture. This higher microbial tolerance for butyl lactate compared to other short chain butyl esters has also been observed in *E. coli* (data not shown). An explanation for this may be that its higher aqueous solubility compared to the other butyl esters investigated in this work, it is less likely to interact with and disrupt membrane function.

Ultimately, the toxicity tests done in this work suggest that nonpolar medium/long chain butyl esters are the best candidates as end-products for this clostridial production platform in terms of host tolerance. However, further considerations need to be made, including the availability of an AAT enzyme that possesses the requisite substrate specificity to make these compounds, and the absence of any endogenous mechanisms capable of breaking down the end-product ester to its alcohol and acid components. While these butyl esters possess intrinsic toxicity, the impact on growth rate observed in this work does not differentiate between inherent toxicity and toxicity that may arise from the alcohol and acid by-products that result from possible ester hydrolysis occurring either spontaneously or enzymatically by endogenous esterases or lipases.

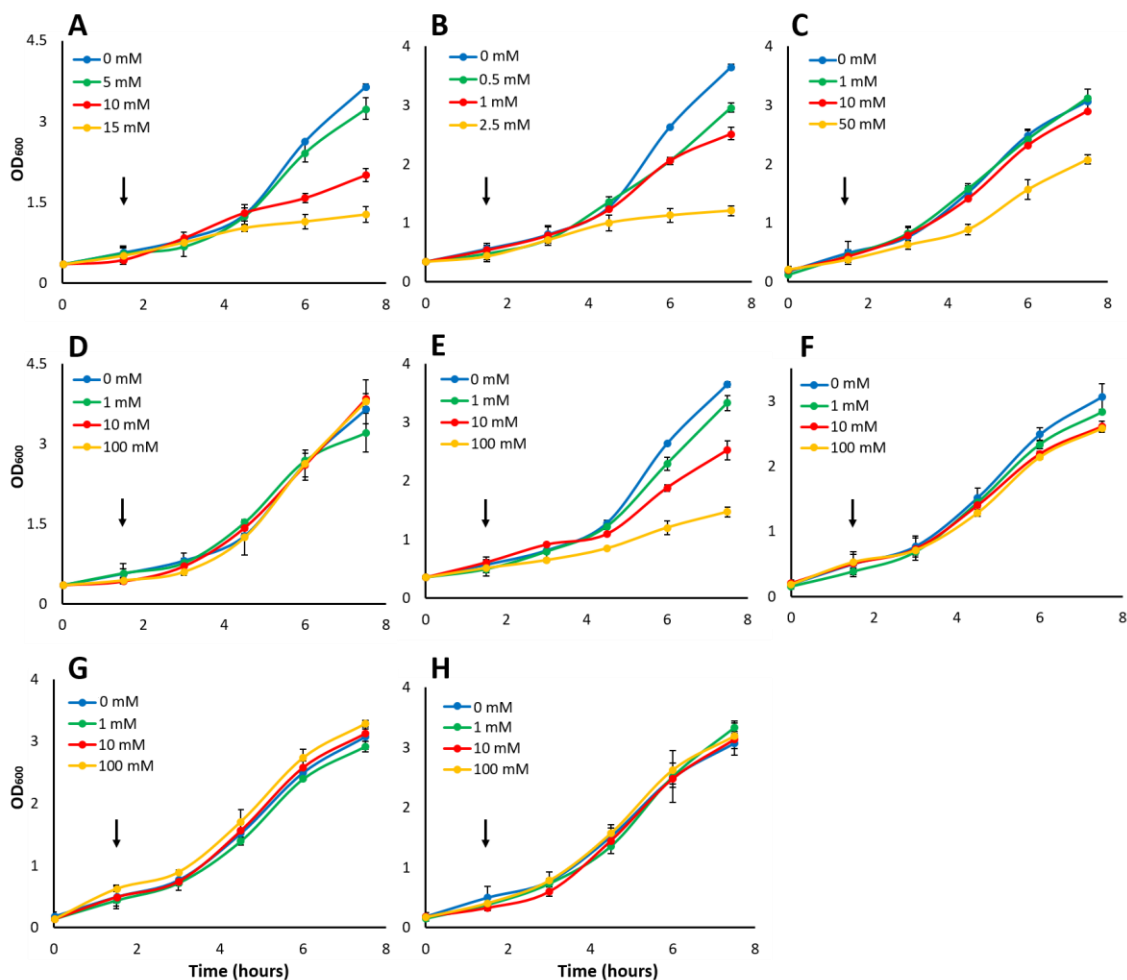


Figure A. 2 Growth of wild type *C. acetobutylicum* exposed to varying concentrations of **A** butyl acetate, **B** butyl acrylate **C** butyl lactate, **D** butyl laurate, **E** dibutyl succinate, **F** butyl stearate, **G** dibutyl maleate, **H** dibutyl sebacate. Cultures were grown at 32°C and 250 rpm and the OD₆₀₀ readings were taken every 90 minutes for 8 hours. Ester feeding was done 1.5 hours after first reading (shown with black arrow). Data are the mean \pm standard deviation from two biological replicates.

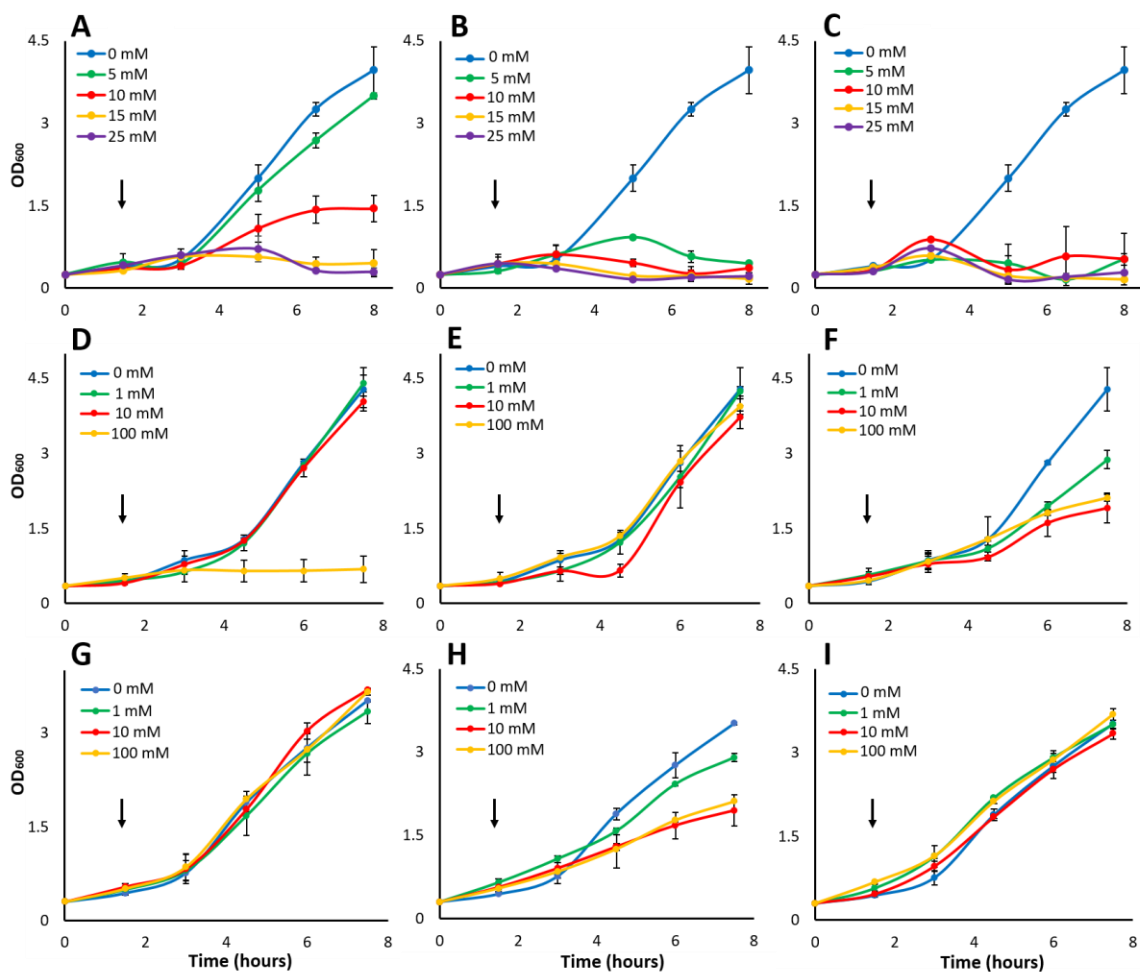


Figure A. 3 Growth of wild type *C. saccharoperbutylacetonicum* exposed to varying concentrations of **A** butyl acetate, **B** butyl acrylate, and **C** butyl butyrate, **D** butyl lactate, **E** butyl laurate, **F** dibutyl succinate, **G** butyl stearate, **H** dibutyl maleate, **I** dibutyl sebacate. Cultures were grown at 32°C and 250 rpm and the OD₆₀₀ readings were taken every 90 minutes for 8 hours. Ester feeding was done 1.5 hours after first reading (shown with black arrow). Data are the mean \pm standard deviation from two biological replicates.

Table A. 2 List of butyl and dibutyl esters and their respective LogP_(ow) values.

Ester	LogP _(ow) ^a
Butyl acetate	1.82
Butyl acrylate	2.36

Butyl butyrate	2.68
Butyl lactate	1.16
Butyl laurate	6.51
Butyl stearate	8.82
Butyl oleate	8.68
Dibutyl succinate	2.88
Dibutyl maleate	3.12
Dibutyl sebacate	5.97

^a values sources from the EPA (United States Environmental Protection Agency) website at: www.epa.gov

A.2.2 Identification and characterization of putative esterases/lipases in *C. saccharoperbutylacetonicum* and *C. acetobutylicum*

In addition to the innate microbial toxicity these butyl esters may exhibit, an additional concern when developing a clostridial platform for the production for these compounds is their potential *in situ* hydrolysis by endogenous enzymes. Not only could butyl ester hydrolysis possibly result in increased culture toxicity due to the accumulation of breakdown products, but it could also result in end-product loss. Hydrolysis may occur either spontaneously or enzymatically; the latter could be addressed in a microbial system by identifying and potentially knocking out/down this activity. Hydrolysis of carboxylic acid esters is typically carried out by two classes of enzyme; either an esterase or a lipase. These two enzyme classes can be differentiated based on their substrate specificity, whereas esterases preferentially hydrolyse water soluble short-chain esters ($\leq C8$), lipases possess a much broader substrate range that is dictated primarily by substrate solubility (Fojan *et al.*, 2000). As such, lipases generally hydrolyse long-chain esters ($\geq C8$) and triglycerides that display low aqueous solubility, however they can also have activity for short chain esters should their concentration in solution exceed their solubility. Both carboxyl esterases and lipases, however, generally display quite promiscuous substrate

specificity, which can extend beyond carboxyl esters, making it difficult to classify putative hydrolases based on substrate specificity alone (Fojan *et al.*, 2000; Bornscheuer *et al.*, 2002; Chahinian and Sarda 2009; Glogauer *et al.*, 2011). Thus, categorising these enzymes also relies heavily on sequence analysis and the identification of specific conserved features that are unique to esterases/lipases, although this can be challenging in its own right as these enzymes generally display enormous sequence diversity (Martinez-Martinez *et al.*, 2013). However, the majority of them do possess common features, such as the conserved consensus sequence motifs: G-X-S-X-G (where X may represent any amino acid) which is present in the majority of carboxyl esterases and lipases, or G-D-S-L which is present in some as well (Arpigny and Jaeger, 1999; Bornscheuer *et al.*, 2002; Chepyshko *et al.*, 2012). To date, no carboxyl esterases or lipases have been characterized from any solventogenic species of clostridia. Recently, however, the genomes of both *C. acetobutylicum* or *C. saccharoperbutylacetonicum* have been sequenced, which facilitated our search for potential candidates in each strain. To do so a number of enzymes were identified that had been categorised as ester hydrolases in the NCBI (National Center for Biotechnology Information) database, and then analysed for the presence of identifying conserved features. From this, six putative esterases/lipases were identified from *C. saccharoperbutylacetonicum*, and three from *C. acetobutylicum* that were probably active towards carboxyl esters. These proteins have been annotated in this work as: Cs2848, Cs4859, Cs5680, Cs2944, Cs2130, Cs5781, Ca2917, Ca1962, and CaLipG, with the first two letters representing the host strain. Each putative esterase/lipase has a G-X-S-X-G consensus sequence, with the exception of Cs5680, which had been predicted to be an ester hydrolase regardless of it lacking this identifying consensus sequences. Table A.3 shows the primary sequence length of each of the nine proteins, as well as their respective consensus sequence. It is evident that the length of each putative ester hydrolase is quite variable. Additionally, the amino acid sequence identity between each protein was quite low – with a range between 11.6% to 28.6% identity between these nine enzymes (data not shown). This variability of sequence length and sequence diversity is common amongst esterases and lipases (Martinez-Martinez *et al.*, 2013).

Table A. 3 List of putative esterases/lipases from *C. saccharoperbutylacetonicum* and *C. acetobutylicum*. The length of each gene sequence, and the amino acid residues that constitute the consensus sequence for each esterase/lipase are indicated.

Gene	Length (aa)	Consensus sequence (G-X-S-X-G)
<i>C. saccharoperbutylacetonicum</i>		
Cs2848	244	GHSMG
Cs4859	511	GQSAG
Cs5680	241	-
Cs2944	379	GTSAG
Cs2130	314	GDSAG
Cs5781	264	GYSMG
<i>C. acetobutylicum</i>		
Ca2917	273	GFSAG
Ca1962	243	GYSLG
CaLipG	300	GASMG

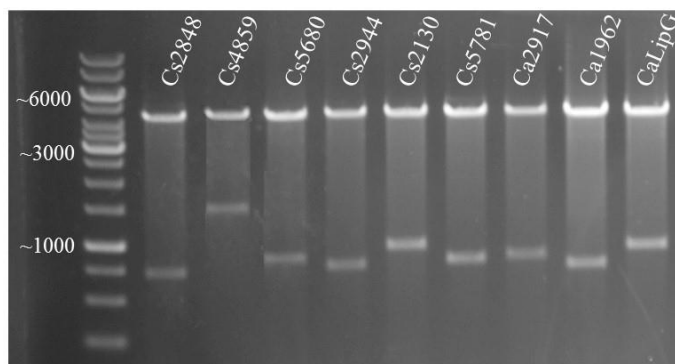


Figure A. 4 A 1% agarose gel electrophoresis showing the restriction digest of each of the cloned constructs containing genes encoding putative esterases/lipases from either *C.*

saccharoperbutylaceticum or *C. acetobutylicum*. Digestion was carried out using *Nde*I and *Bam*HI. The size of the bands (in bp) is indicated to the left of the ladder.

Each gene was amplified from the genomic DNA of either *C. acetobutylicum* or *C. saccharoperbutylaceticum* with gene specific primers that contained *Nde*I and *Xho*I restriction sites – on the forward and reverse primers, respectively – to insert each gene into the multiple cloning site (MCS) upstream of a 6xHis tag, under the control of the strong rhamnose inducible promoter in the *E. coli* expression vector pJOE2792. Figure A.4 shows the electrophoretogram of the fragments from a restriction digestion of each successfully cloned construct. Subsequently, each putative esterase/lipase was expressed in *E. coli* and the enzyme was purified using a cobalt resin for *in vitro* activity assays to determine substrate specificity and chain length preference. The activity assay used in this work was a colorimetric assay using *p*-nitrophenol esters of increasing acid chain length from C2:0-C14:0 as the substrate. This was designed to determine whether any of these enzymes displayed hydrolytic activity and, if so, what their acyl chain length specificity was. From this information, their role as either an esterase or lipase could be deduced, narrowing down which of these enzymes could hydrolyse any of our desired end-product butyl ester. Initially, activity of each of these putative esterases/lipases was quantified for *p*-nitrophenol esters with acid chain lengths of C2:0, C4:0 and C8:0, to identify potential esterase activity for shorter chain substrates. Table A.4 shows the kinetic data for each of the nine enzymes for these three substrates; here it can be seen that each of the putative esterases/lipases displayed some degree hydrolysis activity, although their chain length substrate specificity varied. All of the putative hydrolases had activity towards the *p*-nitrophenyl acetate (C2:0) and -butyrate (C4:0) substrates, with the exception of Cs2130, which was only able to hydrolyse *p*-nitrophenyl acetate chain with a relatively low affinity for this substrate when comparing its K_M to that of the other enzymes. As an example of the data from which these kinetics were derived, figures A.5 and A.6 shows the Michaelis-Menton and Hanes-Woolf plots for two of the putative esterases/lipases, Cs4859 and CaLIPG, for *p*-nitrophenyl acetate (A) and -butyrate (B) substrates. Interestingly, the majority of the putative carboxyl hydrolases either did not have activity for – or had low activity for – *p*-nitrophenyl octanoate (C8:0). However, as mentioned above, the majority of hydrolase enzymes display broad substrate range (Bornscheuer *et al.*, 2002). This would suggest either that these enzymes are less

promiscuous than most of the members of this enzyme family, or that perhaps carboxyl esters are not their primary substrate.

Table A. 4 *In vitro* activity of the putative esterase/lipase enzymes for the hydrolysis of *p*-nitrophenol acetate, -butyrate, and -octanoate. Kinetic parameters were determined using a colorimetric assay. Data are the mean \pm standard error from two replicates.

enzyme	<i>p</i> -NP-acetate (C2)	<i>p</i> -NP-butyrate (C4)	<i>p</i> -NP-octanoate (C8)
Cs2848	$V_{\max}^a: 21.6 \pm 3.1$ $K_M^b: 2.0 \pm 0.5$	$V_{\max}: 17.4 \pm 2.7$ $K_M: 1.7 \pm 0.6$	NA
Cs4859	$V_{\max}: 129.5 \pm 7.1$ $K_M: 5.1 \times 10^{-2} \pm 1.2 \times 10^{-2}$	$V_{\max}: 187.8 \pm 65.6$ $K_M: 0.3 \pm 0.2$	Trace activity
Cs5680	$V_{\max}: 121.5 \pm 15.1$ $K_M: 7.1 \times 10^{-2} \pm 2.0 \times 10^{-3}$	$V_{\max}: 61.2 \pm 2.6$ $K_M: 2.7 \times 10^{-3} \pm 4.5 \times 10^{-3}$	$V_{\max}: 28.4 \pm 5.1$ $K_M: 1.8 \pm 0.5$
Cs2944	$V_{\max}: 36.0 \pm 5.7$ $K_M: 2.85 \pm 0.7$	$V_{\max}: 27.2 \pm 3.8$ $K_M: 1.6 \pm 0.4$	NA
Cs2130	$V_{\max}: 149.8 \pm 71.4$ $K_M: 7.2 \pm 5.5$	NA	NA
Cs5781	$V_{\max}: 58.7 \pm 21.4$ $K_M: 8.3 \pm 4$	$V_{\max}: 113.8 \pm 8.6$ $K_M: 1.3 \pm 0.23$	$V_{\max}: 69.1 \pm 6.4$ $K_M: 3.6 \times 10^{-3} \pm 0.01$
Ca2917	$V_{\max}: 53.3 \pm 3.4$ $K_M: 0.18 \pm 2.6 \times 10^{-2}$	$V_{\max}: 14.1 \pm 1.2$ $K_M: 3.2 \pm 0.4$	Trace activity
Ca1962	$V_{\max}: 21.6 \pm 3.1$ $K_M: 2.0 \pm 0.5$	$V_{\max}: 17.4 \pm 2.7$ $K_M: 1.7 \pm 0.6$	NA
CaLipG	$V_{\max}: 9.1 \pm 0.8$ $K_M: 6.7 \times 10^{-2} \pm 1.7 \times 10^{-2}$	$V_{\max}: 28.8 \pm 1.4$ $K_M: 0.1 \pm 1.5 \times 10^{-2}$	NA

^a $\mu\text{mol}/\text{min}/\text{mg}$, ^b mM, NA – no activity

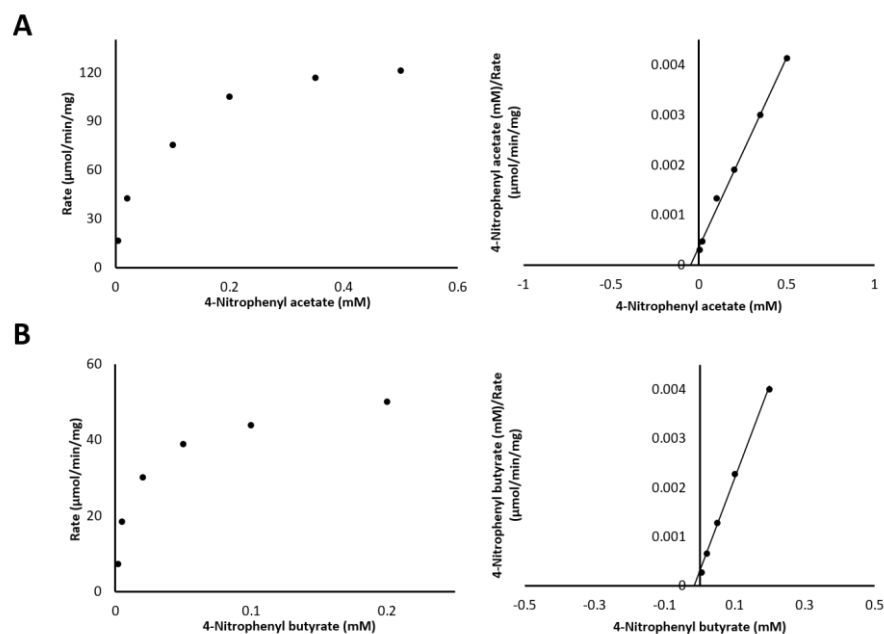


Figure A. 5 Michaelis-Menton (left) and Hanes-Woolf (right) plots of *C. saccharoperbutylacetonicum* Cs4859 esterase activity using **A** *p*-nitrophenyl acetate and **B** *p*-nitrophenyl butyrate as substrate. Assays were carried out at 25°C and *p*-nitrophenol concentration was measured at 400 nm. [S] is shown in mM and rate in $\mu\text{mol/min/mg}$ protein.

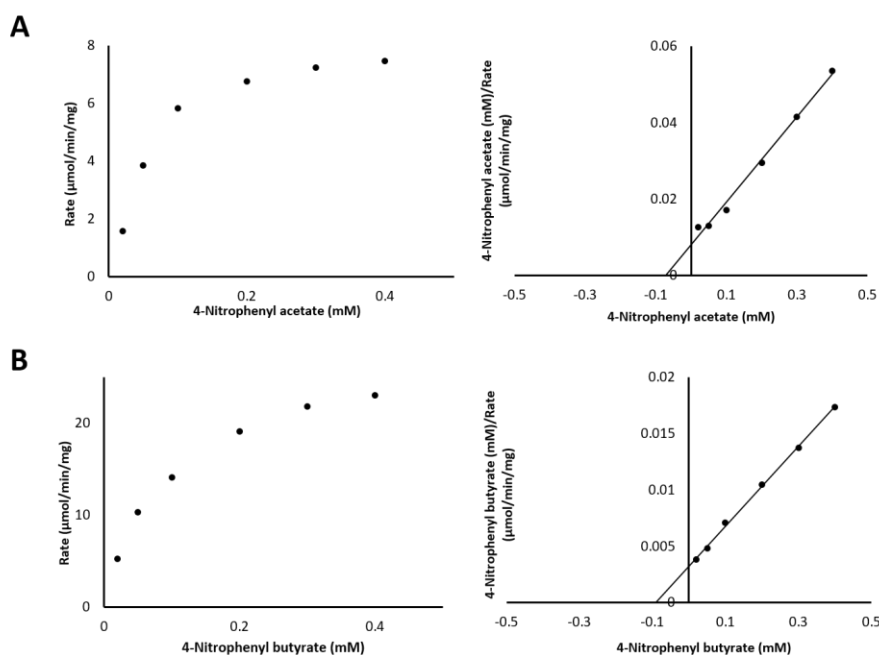


Figure A. 6 Michaelis-Menton (left) and Hanes-Woolf (right) plots of *C. acetobutylicum* CaLIPG esterase activity using **A** *p*-nitrophenyl acetate and **B** *p*-nitrophenyl butyrate as substrate. Assays were carried out at 25°C and 400 nm. [S] is shown in mM and rate in $\mu\text{mol/min/mg}$ protein.

Those enzymes that did display quantifiable or trace activity for the octanoate chain substrate (Cs4859, Cs5680, Cs5781, and Ca2917) were further characterized for activity towards long-chain *p*-nitrophenyl esters (C14:0 and C16:0) to determine whether or not their activities may classify them as lipases. Interestingly, CaLipG had not had activity for the longer octanoate chain, despite its annotated name suggesting it has lipase activity. Each of the four enzymes displayed some activity for hydrolysing both the *p*-nitrophenyl myristate or -palmitate substrates when exposed to a concentration of 500 μ M (Table A.5). This suggests that these enzymes may be categorised as lipases rather than esterases as it is common for a lipase enzyme to display hydrolytic activity towards shorter chain substrates in addition to the longer chains – as observed in this work with these four enzymes (Chahinian and Sarda 2009).

Table A. 5 Activity (μ mol *p*-NP/mg/min) of putative lipases using 500 μ M *p*-nitrophenyl myristate, and –palmitate. Data are the mean \pm standard error from three replicates.

	Specific Activity (μ mol <i>p</i> -NP/mg/min)	
	<i>p</i> -NP-myristate (C14)	<i>p</i> -NP-palmitate (C16)
Cs4859	0.364 + 6.7x10 ⁻²	0.066 + 5.1x10 ⁻³
Cs5680	0.427 + 2.3x10 ⁻²	0.126 + 6.8x10 ⁻³
Cs5781	0.055 + 1.5x10 ⁻²	0.077 + 1.6x10 ⁻²
Ca2917	0.101 + 3.9x10 ⁻²	0.254 + 6.7x10 ⁻³

Of the nine enzymes investigated here, all displayed some carboxyl ester hydrolytic activity, with the majority preferentially hydrolysing shorter chain esters of chain length C2:0 and C4:0. Generally, hydrolases such as esterases and lipases display broad substrate specificity as they are involved in a wide spectrum of biocatalysed processes (Busto *et al.*, 2010), which does not correlate with the relatively narrow range of activity seen here for these putative carboxyl esterases/lipases. Possible explanations for these discrepancies may be:

- a) That these enzymes are a different class of hydrolase that possesses some activity towards carboxyl esters when the substrate present at high concentrations, such as in an *in vitro* environment.
- b) That these enzymes display a substrate specificity that has not been looked at in this work, such as preferred activity towards saturated or branched acyl chains.
- c) That the assay used is not representative enough of the enzyme's true cellular substrate. While *p*-nitrophenyl esters provide an excellent tool for quick and high throughput assessment of hydrolytic activity, they are structurally distinct from the true carboxyl ester substrates found in the cell. The broad substrate specificities of Cs4859, Cs5680, Cs5781, and Ca2917, however, was more akin to true carboxyl esterase/lipase activity, and as such, these enzymes would be the most probable candidates responsible for any detrimental butyl ester hydrolysis in a clostridial system of the nine enzymes examined here.

A.2.3 Investigation of *in vivo* hydrolysis of fed butyl and dibutyl esters in cultures of *C. saccharoperbutylacetonicum* and *C. acetobutylicum*

To determine whether or not there is any *in vivo* hydrolysis of our desired end-product butyl esters in either clostridial strain, the culture supernatant of both *C. saccharoperbutylacetonicum* and *C. acetobutylicum* was extracted and analysed after 8 hours of incubation in the presence of 10 mM of an exogenously fed butyl ester. This was done to investigate whether after internalisation of a given butyl ester, it was being hydrolysed by internal clostridial enzymes, followed by secretion of the resulting alcohol and acid into the culture supernatant. The culture supernatant was analysed as the fatty acids and alcohols should readily diffuse out of bacterial cells (Liu *et al.*, 2012; Wen *et al.*, 2016). What was observed when examining the supernatant fatty acids was the presence of stearic acid (C18:0) in cultures of both clostridial strains grown in the presence of butyl stearate, as well as a small amount of lauric acid (C12:0) in cultures of *C. saccharoperbutylacetonicum* grown in the presence of butyl laurate. Hydrolysis products were not observed in cultures exposed to any of the other butyl esters examined, nor in the wild type controls for each strain (Figure A.7). This suggests that that both strains most likely contain

a lipase(s) with substrate specificity for hydrolysing long chain saturated esters such as butyl stearate and –laurate, as was observed in culture, but not unsaturated esters such as butyl oleate (C18:1), which possesses a double bond. Previously, nine potential esterases/lipases from *C. saccharoperbutylacetonicum* and *C. acetobutylicum* were identified, four of which demonstrated activity towards longer chain ester (Cs4859, Cs5680, Cs5781, and Ca2917), making them potential candidates for this *in vivo* hydrolysis. While *in vitro*, these four enzymes were also able to hydrolyse short chain esters, the lack of observed *in vivo* hydrolysis of these substrates is not wholly unexpected as lipases are able to work on shorter chain substrates only when present in excess – as is most likely not the case here. An additional possibility is that the observed hydrolysis of butyl laurate and -stearate in culture is being carried out by a yet uncharacterised enzyme. To further determine whether or not one of the nine esterase/lipases identified in this work is responsible for the observed hydrolysis we could further examine the transcript levels of each enzyme in cultures that are grown in the presence of either butyl laurate or -stearate. It may be the case that the presence of these butyl esters results in the upregulation of the responsible lipase(s) genes, resulting in increased hydrolysis. Interestingly, while both butyl laurate and -stearate seem to be broken down in culture, they both exhibit low toxicity at concentrations as

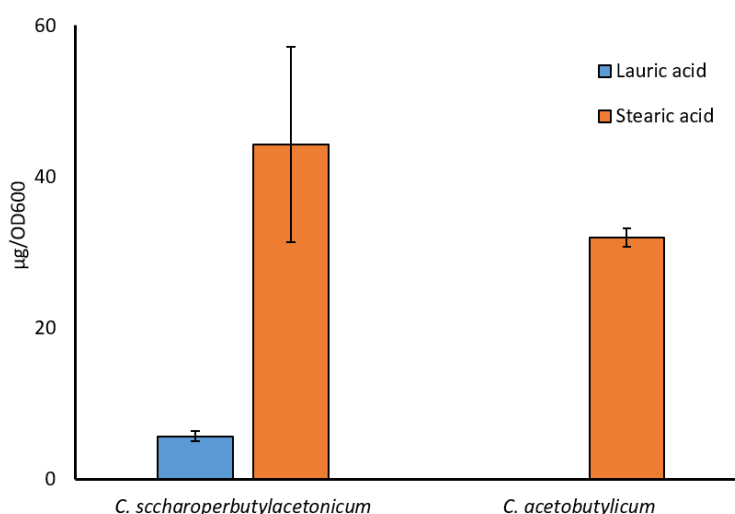


Figure A. 7 Quantification of lauric acid (C12:0) and stearic acid (C18:0) found in the supernatant of cultures of *C. saccharoperbutylacetonicum* and *C. acetobutylicum* grown in the presence of exogenously fed butyl laurate and butyl stearate, respectively.

high at 100mM. While this low toxicity may be a true reflection of clostridia's tolerance for these esters and their breakdown products, an alternative explanation for this could be that due to their size, these butyl esters are not able to pass across the cell membranes into the cell, and instead, are being broken down by extracellular lipase activity. Should this be the case, the inability to cross the cell membranes would be an undesirable quality of our end point ester product as it would increase the costs of product extraction from the culture. A possible strategy to investigate whether or not these esters are able to move across the membrane would be to heterologously express an internal lipase in cultures fed long chain butyl esters – as was done in chapter 3. Should the esters be internalized, they will be hydrolysed by the lipase and the resulting breakdown products may be quantified by chromatography.

A.3 Conclusion

Ultimately, the results obtained from this work would suggest that clostridia would make a good platform for the production of commercially valuable medium chain butyl esters such as dibutyl maleate, dibutyl succinate, to some extent butyl laurate, as well as other medium chain esters not yet investigated. The reason for this is that these medium chain butyl esters display low microbial toxicity in cultures of solventogenic clostridia when present in high concentrations, and also are minimally hydrolysed in culture. Additionally, previous work done in chapter 3 demonstrated that medium chain butyl esters, such as butyl octanoate and decanoate, are able to pass across the cell membranes in *E. coli* to the extracellular milieu, which may be the same for clostridia. Therefore, this would indicate that medium chain butyl esters strike a happy balance between low culture toxicity, ability to pass across the cell membranes for ease of downstream recovery, and minimal enzymatic hydrolysis by esterase/lipase activity. The next key step in developing solventogenic clostridia as a platform for butyl ester production would be to identify alcohol acyltransferase (AAT) enzymes capable of producing these desired end products and expressing them in a solventogenic strain of clostridia.